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Investigations of $GABA_A$ Receptor Phosphorylation and Receptor-Protein Interactions

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To Jon

Abstract

$GABA_A$ receptors mediate the majority of fast synaptic inhibition in the adult mammalian brain, and play a critical role in controlling neuronal excitability during development. The functional properties and stability of cell-surface $GABA_A$ receptors are key determinants of the efficacy of GABAergic neurotransmission. I therefore employed a broad spectrum of biochemical, and cell and molecular biological techniques to identify molecular interactions that may be involved in regulating the activity and number of $GABA_A$ receptors at the neuronal surface.

In this thesis, I identified a novel interaction between the $GABA_A$ receptor and the calcium/calmodulin-dependent protein kinase II (CaMKII). Affinity-purification assays revealed that CaMKII forms a native complex with the $GABA_A$ receptor in brain, and that the kinase binds non-selectively to the major intracellular domain (ICD) of various $GABA_A$ receptor subunits. The interaction between CaMKII and the receptor β subunits was found to be dependent upon phosphorylation of the kinase at T286, but appeared to be independent of subunit phosphorylation. Furthermore, a CaMKII binding site was identified in the ICD of the receptor β subunits, between residues 304 and 323.

Further work demonstrated that CaMKII selectively phosphorylates the ICDs of the receptor β and γ subunits, and identified protein phosphatases that dephosphorylate the receptor β3 subunit at the CaMKII sites. Depolarisation of cultured immature cortical neurons was found to increase the level of enzymatically-active CaMKII that associates with the ICD of the receptor β3 subunit, and to trigger CaMKII-dependent phosphorylation of the receptor β3 subunit at both the S408 and S409 residues.

Work presented in this thesis also identified multivalent interactions between $GABA_A$ receptor γ subunits and the AP2 adaptor complex, a major component of the endocytic machinery. The ICD of each receptor γ subunit was found to interact with the native AP2 complex from brain, and to bind selectively and directly to the μ2-adaptin of AP2. Two distinct binding sites for μ2-adaptin were identified in the ICD

of the γ2S subunit. The N-terminal half of the ICD was found to interact with sub-domain B of μ2-adaptin via a putative basic-rich motif. In contrast, the C-terminal half of the ICD was found to bind sub-domain A of μ2-adaptin, likely via a tyrosine-based motif. Both μ2-adaptin binding motifs were found to be common to all the γ subunit isoforms.

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Abbreviations

A	alanine
A_x	absorbance at x nm
AKAP	A-kinase anchoring protein
AKAP-79/150	AKAP protein of 79/150 kDa
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
Amp	ampicillin
AP	alkaline phosphatase
AP2	adaptor-binding protein 2
APS	ammonium Persulphate
ARF	ADP-ribosylation factor
AS	Angelman syndrome
ATP	adenosine triphosphate
BAPTA	1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid
BAPTA-AM	as for 'BAPTA' but membrane permeant
BCA	bicinchoninic protein assay
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BDNF	brain-derived neurotrophic factor
BIG2	brefeldin A-inhibited guanine nucleotide-exchange protein type 2
BSA	bovine serum albumin
C	cysteine
CACA	*cis*-4-aminocrotonic acid
CaM	calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase type 2
CaMKIIα	CaMKII alpha subunit
CaMKIIβ	CaMKII beta subunit
CaMKII-TA	autophosphorylation-deficient CaMKII (T286A point mutant)
CaMKII-TD	constitutively active CaMKII (T286D point mutant)
CaMKII-WT	wild type CaMKII

CAMP	*cis*-2-aminomethyl-cyclopropane carboxylic acid
cAMP	cyclic adenosine monophosphate
CCC	cation-chloride co-transporter
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
Chloroamp	chloramphenicol
CNS	central nervous system
COS-7	African green monkey kidney
cpm	counts per min
cSrc	cellular Src
D	aspartate
DARRP-32	dopamine and cAMP-regulated phosphoprotein of 32-kDa
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DG	1,2-dioleoyl-sn-glycerol
dGTP	2'-deoxyguanosine 5'-triphosphate
DIV	days *in vitro*
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOC	deoxycholate
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
E	glutamate
E17	embryonic day 17
E_{Cl}	equilibrium potential of chloride
ECL	enhanced chemiluminescence
E. Coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
E_{GABA}	equilibrium potential for GABA
EGFR	epidermal growth factor receptor
EGTA	ethylenebis(oxonitrilo)tetraacetate

ER	endoplasmic reticulum
F	phenylalanine
FBS	foetal bovine serum
γ2S	γ2 short subunit
γ2L	γ2 long subunit
GABA	gamma-aminobutyric acid
$GABA_A$ receptor	type-A GABA receptor
$GABA_B$ receptor	type-B GABA receptor
$GABA_C$ receptor	type-C GABA receptor
GABARAP	$GABA_A$ receptor-associated protein
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAT	GABA transporter
GATE-16	Golgi-associated transport enhancer of 16 kDa
GEC-1	GABARAP-like protein type 1
GluR2	AMPA-type glutamate receptor subunit type 2
GODZ	Golgi-specific DHHC zinc finger protein
GPCR	G-protein coupled receptor
G-protein	guanine nucleotide binding proteins
GRIF-1	$GABA_A$ receptor interacting factor type-1
GRIP1	glutamate receptor interacting protein type 1
GST	glutathione-S-transferase
h	hour
HAP-1	huntingtin-associated protein type-1
HCl	hydrochloric acid
HEK-293	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
5-HT-2 receptor	5-hydroxytryptamine type 2 receptor
5-HT-3 receptor	5-hydroxytryptamine type 3 receptor
5-HT-4 receptor	5-hydroxytryptamine type 4 receptor

ICD	major intracellular domain
Ile	isoleucine
IN	input
IP_3	inositol-1,4,5-triphosphate
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
IPTG	isopropylthio-b-D-galactoside
K	lysine
KCC2	K^+-Cl^- co-transporter 2
kDa	kilodalton
KN93	*N*-[2-[[[3-(4-Chlorophenyl)-2-propenyl]methylamino] methyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzenesulphonamide
KO	knockout
L	leucine
LB	Luria-Bertani
Leu	leucine
LTD	long-term depression
LTP	long-term potentiation
M	methionine
MAP1X	microtubule-associated protein 1X
Met	methionine
mGluR	metabotropic glutamate receptor
min	minute
mIPSC	miniature IPSC
mRNA	messenger RNA
n	number of experiments
N	asparagine
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitro-blue tetrazolium chloride
NKCC1	Na^+-K^+-$2Cl^-$ co-transporter 1

NMDA	N-methyl D-aspartate receptors
NP40	nonidet P-40
NSF	N-ethylmaleimide-sensitive factor
OD	optical density
P	proline
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Phe	phenylalanine
PIP2	phosphatidylinositol biphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PKA	cAMP-dependent protein kinase
PKB	protein kinase B (also known as Akt)
PKC	calcium/phospholipid-dependent protein kinase
PKG	cGMP-dependent protein kinase
PLIC-1	protein that links integrin-associated protein with the cytoskeleton type-1
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase type 1
PP2A	protein phosphatase type 2A
PP2Ac	catalytic subunit of PP2A
PP2B	calcium/CaM-dependent phosphatase 2B/protein phosphatase type 2B (also known as calcineurin)
PP2C	protein phosphatase type 2C
PRIP-1	phospholipase C-related catalytically inactive protein type 1
PSEL	L-a-phosphatidylserine
PSD	postsynaptic density
PSD-95	postsynaptic density protein of 95 kDa
PTM	post-translational modification
PVDF	polyvinylidene fluoride
Q	glutamine
R	arginine
RACK-1	receptor for activated C-kinase type 1

RNA	ribonucleic acid
rpm	revolutions per minute
S	serine
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sem	standard error of the mean
SNAP25	synaptosomal-associated protein of 25-kDa
sulfo-MBS	*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester
T	threonine
TASK-1	TWIK-related acid-sensitive potassium channel type 1
TAE	tris-acetate EDTA
TBS	tris buffered saline
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethyl-ethylene diamine
temp	temperature
THIP	4,5,6,7-tetrahydroisoxazolo-pyridin-3-ol
TM	transmembrane
TMX	transmembrane domain X
TPMPA	(1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid
UT	untransfected/mock-transfected
V	valine
VDCCs	voltage-dependent calcium channels
VIAAT	vesicular inhibitory amino acid transporter
W	tryptophan
WT	wild type
Y	tyrosine

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CHAPTER 1

Introduction

PART ONE

1.1 The Identification and Synthesis of the Neurotransmitter GABA

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the adult mammalian brain. This neutral amino acid was originally identified in mammalian brain in 1950 (Awapara *et al.*, 1950; Roberts and Frankel 1950), and by the 1970s, several lines of evidence had culminated in the recognition of GABA as an inhibitory neurotransmitter (reviewed in Owens and Kriegstein, 2002 and Bowery and Smart, 2006; see also Krnjevic and Schwartz, 1967; Elliot and Florey, 1956; Bazemore *et al.*, 1956; Basemore *et al.*, 1957; Kravitz *et al.*, 1963; Kravitz and Potter, 1965; Otsuka *et al.*, 1966; Obata, 1972; Kuffler and Edwards 1958; Bloom and Iversen, 1971). GABA is synthesised in the terminals of inhibitory neurons from the amino acid glutamate in a reaction catalysed by glutamic acid decarboxylase (GAD), of which there are at least two forms, GAD65 and GAD67 (Roberts and Frankel 1950; Martin and Rimvall, 1993; Erlander *et al.*, 1991; Owens and Kriegstein, 2002). Once produced, GABA is transported into synaptic vesicles by a vesicular inhibitory amino acid transporter (VIAAT) ready for calcium-dependent exocytosis (Owens and Kriegstein, 2002). However, GABA is also subject to non-vesicular release by the reverse action of plasma membrane GABA transporters (GATs) (Richerson and Wu, 2003; Cavelier *et al.*, 2005; Owens and Kriegstein, 2002). GABA is predominantly released from interneurons, and exerts its effects by binding to plasma membrane GABA receptors (see Sections 1.2 and 1.3). The uptake of GABA into nearby neurons and glia by GATs terminates GABAergic transmission, and GABA is subsequently metabolised in a reaction catalysed by GABA transaminase, which transfers the amino group from GABA to α-oxoglutarate to yield the GABA precursor, glutamate, and succinic semialdehyde (Iversen and Neal, 1968; Owens and Kriegstein, 2002; Cherubini and Conti, 2001).

1.2 GABA Activates GABA Receptors to Mediate Neuronal Inhibition or Excitation

GABA receptors are expressed in most neurons, and are divided into two main classes: the ionotropic $GABA_A$ and $GABA_C$ receptors, which mediate an early fast

response to GABA, and the metabotropic $GABA_B$ receptors, which mediate a later slower response to GABA.

$GABA_A$ receptors are ligand-gated hetero-oligomeric anion channels. Activation of these receptors is most effective following binding of two agonist molecules (Baumann *et al.*, 2003), and results in increased membrane permeability to chloride and bicarbonate ions (Kaila, 1994). In mature neurons, this usually results in a net inward flow of anions and hyperpolarisation, which reduces neuronal excitability. This $GABA_A$ receptor-mediated inhibition can be described as either 'phasic' or 'tonic' (reviewed in Farrant and Nusser, 2005). Phasic (fast synaptic) inhibition results from the release of GABA from presynaptic vesicles and the subsequent transient activation of postsynaptic $GABA_A$ receptors, which are clustered opposite the release site (Farrant and Nusser, 2005). The resultant inhibitory postsynaptic current (IPSC) is characterised by the magnitude and duration of the GABA transient and the functional properties, number and location of the $GABA_A$ receptors, and their exposure to modulators (Mody and Pearce, 2004; Cherubini and Conti, 2001). In addition, a 'slower' type of phasic inhibition has been described, which involves spillover of GABA from the synaptic cleft and transient activation of spatially distinct $GABA_A$ receptors, such as those at perisynaptic or extrasynaptic locations (Wei *et al.*, 2003; Rossi and Hamann, 1998; Mody and Pearce, 2004; Kullmann *et al.*, 2005; Nusser *et al.*, 1998a). In contrast to phasic inhibition, tonic inhibition involves the persistent activation of high-affinity extrasynaptic $GABA_A$ receptors by ambient concentrations of GABA (Mody and Pearce, 2004). GABA-mediated tonic conductances have been identified in several types of neurons (Brickley *et al.*, 1996, 2001; Farrant and Nusser, 2005; Nusser and Mody, 2002). In cerebellar granule cells, the tonic conductance is dependent upon the expression of $GABA_A$ receptor $\alpha 6$ and δ subunits (see Section 1.3) (Brickley *et al.*, 2001). The importance of this conductance has been revealed in studies of transgenic animals that lack these subunits, which have shown that it is compensated by up-regulation of a TASK-1-like potassium conductance (Brickley *et al.*, 2001).

Whilst GABA is most commonly recognised as an inhibitory neurotransmitter, $GABA_A$ receptor-mediated neuronal excitation has now been described both in immature neurons and some mature neurons (Owens *et al.*, 1996, 1999; Ben-Ari *et al.*, 1989; Ben-Ari, 2002; Chen *et al.*, 1996; Wang *et al.*, 2001; Gao and van den Pol, 2001; Stein and Nicoll, 2003). In developing neurons, the equilibrium potential for GABA (E_{GABA}) is more positive than the resting membrane potential of the cell (Stein and Nicoll, 2003). This forms the basis of excitation as activation of $GABA_A$ receptors results in a net outward flow of anions and depolarisation. This phenomenon can cause the firing of an action potential (Wang *et al.*, 2001; Gao and van den Pol, 2001; Chen *et al.*, 1996), in addition to activation of voltage-dependent calcium channels (VDCCs) (Owens *et al.*, 1996) and removal of the magnesium block of the NMDA receptor (Leinekugel *et al.*, 1997). GABA therefore plays a role in controlling neuronal excitability during development, and GABA-mediated signalling has been implicated in a number of developmental processes, such as synapse formation, neurite outgrowth and neuronal migration (Ben-Ari, 2002; Owens and Kriegstein, 2002).

E_{GABA} is largely influenced by the equilibrium potential of chloride (E_{Cl}), as $GABA_A$ receptors are most permeable to this anion (Kaila, 1994; Stein and Nicoll, 2003). Neuronal chloride homeostasis is therefore a critical determinant of E_{GABA}, and hence the excitatory action of GABA in immature neurons. The transmembrane chloride gradient is predominantly controlled by a family of cation-chloride co-transporters (CCCs) (Payne *et al.*, 2003; Mercado *et al.*, 2004). Immature neurons have a high intracellular concentration of chloride, and hence a depolarising value of E_{Cl}. This has been attributed to expression of the inwardly directed Na^+-K^+-$2Cl^-$ co-transporter 1 (NKCC1), which mediates chloride uptake (Yamada *et al.*, 2004; Delpire, 2000; Payne *et al.*, 2003; Stein and Nicoll, 2003). During neuronal maturation, the intracellular concentration of chloride decreases. This is caused in part by progressive down-regulation of NKCC1 (Plotkin *et al.*, 1997) and up-regulation of the outwardly directed K^+-Cl^- co-transporter 2 (KCC2), which extrudes chloride from the cell (Rivera *et al.*, 1999, 2005; Clayton *et al.*, 1998; Lu *et al.*, 1999; Payne *et al.*, 2003; Luhmann and Prince, 1991). These changes are believed to account for the

hyperpolarising shift of E_{Cl} and hence the developmental switch in the action of GABA from excitatory to inhibitory (Rivera *et al.*, 1999; Stein and Nicoll, 2003).

$GABA_A$ receptors contain a number of distinct binding sites that are accessible to various types of extracellular ligands. The GABA-binding site interacts with various $GABA_A$ receptor agonists and antagonists, in addition to the endogenous neurotransmitter. Receptor agonists include the mushroom product, muscimol, and synthetic compounds, such as THIP and isoguvacine (Bormann, 2000; Macdonald and Olsen, 1994; Mehta and Ticku, 1999). Receptor antagonists include the plant alkaloid and convulsant, bicuculline, gabazine (SR95531) and (+)-β-hydrastine (Bormann, 2000; Mehta and Ticku, 1999; Huang and Johnston, 1990). The $GABA_A$ receptor ion channel is blocked by convulsants such as picrotoxinin, which exhibits non-competitive inhibition, and TBPS (Macdonald and Olsen, 1994). $GABA_A$ receptor function is also subject to regulation by zinc ions and changes in pH (Hosie *et al.*, 2003; Krishek *et al.*, 1996; Wilkins *et al.*, 2005).

$GABA_A$ receptors contain distinct modulatory binding sites for a number of centrally active drugs, including benzodiazepines, barbiturates neurosteroids, some anaesthetics and ethanol (reviewed in Macdonald and Olsen, 1994). The binding of an agonist ligand to the benzodiazepine site allosterically enhances the action of GABA, by increasing the opening frequency of the $GABA_A$ receptor channel (Macdonald and Olsen, 1994). Benzodiazepines can mediate anxiolytic, anti-convulsant, muscle relaxant, amnesic, sedative and hypnotic effects, and include agents such as diazepam and lorazepam (Mohler *et al.*, 2002). Studies using knockin mice have begun to relate these therapeutic actions to particular $GABA_A$ receptor subtypes and amino acid residues (Mohler *et al.*, 2002; Rudolph and Mohler, 2004). However, disadvantages of the clinical use of benzodiazepines include symptoms of withdrawal and the development of tolerance and dependence. Inverse benzodiazepine agonists have also been developed, and these have been shown to exert anxiogenic and convulsant effects. The binding of an agonist ligand to the barbiturate site can activate $GABA_A$ receptors directly, and allosterically enhance the action of GABA, apparently by enhancing the mean channel open duration

(Macdonald and Olsen, 1994). Barbiturate drugs can mediate anxiolytic, sedative, hypnotic, anti-convulsant and anaesthetic effects, and include agents such as pentobarbitone, phenobarbitone and thiopentone. However, barbiturates induce tolerance and dependence, and can be lethal when overdosed. Neurosteroids can also activate $GABA_A$ receptors directly and potentiate the effect of GABA (Macdonald and Olsen, 1994). The neurosteroid site binds metabolites of endogenous steroid hormones, such as androsterone and pregnanolone, which have a sedative effect, and synthetic compounds such as the anaesthetic alphaxalone (Macdonald and Olsen, 1994). Several gaseous and intravenous anaesthetics, such as enflurane, halothane and propofol are also believed to act at distinct sites on $GABA_A$ receptors (Mehta and Ticku, 1999; Rudolph and Mohler, 2004).

Like $GABA_A$ receptors, $GABA_C$ receptors are ligand-gated chloride-permeable hetero-pentameric ion channels. Although $GABA_C$ receptors display some structural similarity to the $GABA_A$ receptor (see Section 1.3), they are predominantly expressed in the vertebrate retina and are pharmacologically distinct (Polenzani *et al.*, 1991; Feigenspan *et al.*, 1993; Cutting *et al.*, 1991; Koulen *et al.*, 1997, 1998; Bormann and Feigenspan, 1995). For example, $GABA_C$ receptors exhibit greater sensitivity to GABA than $GABA_A$ receptors, and desensitise more slowly. Furthermore, unlike $GABA_A$ receptors, $GABA_C$ receptors are activated by the agonists CACA and CAMP, and bind the antagonist, TPMPA (Bormann, 2000; Bormann and Feigenspan, 1995). Conversely, the $GABA_C$ receptor is insensitive to the $GABA_A$ receptor antagonist, bicuculline, and $GABA_A$ receptor modulators such as benzodiazepines, barbiturates and neurosteroids (Bormann, 2000).

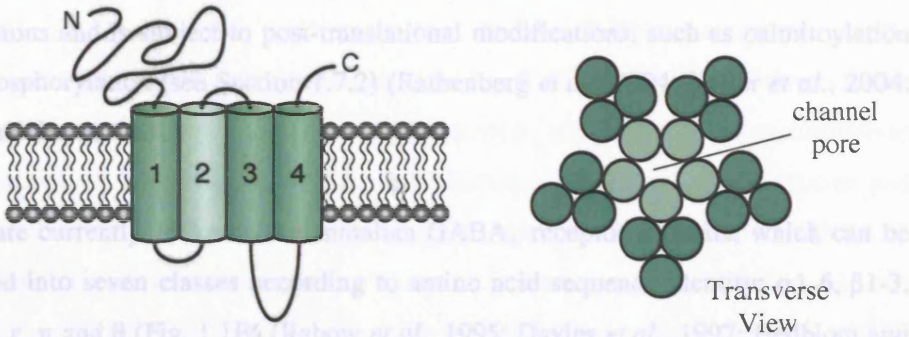
In contrast to the ionotropic $GABA_A$ and $GABA_C$ receptors, which mediate fast synaptic transmission, the metabotropic $GABA_B$ receptor mediates slow inhibitory neurotransmission. The $GABA_B$ receptor exists as a heterodimer and is expressed pre- and postsynaptically. The two constituent subunits, $GABA_{B1}$ and $GABA_{B2}$, were only recently cloned (Kaupmann *et al.*, 1997, 1998; White *et al.*, 1998; Jones *et al.*, 1998), although a number of splice variant isoforms have since been identified for each subunit (Kaupmann *et al.*, 1997; Isomoto *et al.*, 1998; Couve *et al.*, 2000). Each

$GABA_B$ receptor subunit comprises a large extracellular N-terminal region, seven transmembrane (TM) domains and an intracellular C-terminal tail, which contains a coiled-coil domain involved in subunit coupling (Kammerer *et al.*, 1999). Functional $GABA_B$ receptors are trafficked to the plasma membrane in the heterodimeric form (White *et al.*, 1998; Kuner *et al.*, 1999; Couve *et al.*, 2000). The extracellular domain of the $GABA_{B1}$ subunit confers binding to GABA, and the extracellular domain of the $GABA_{B2}$ subunit contains a binding site for an allosteric modulator (Bowery and Smart, 2006). $GABA_B$ receptors decrease neuronal excitability by activating postsynaptic potassium currents and inhibiting pre-synaptic calcium currents via G-protein coupling. Receptor-G-protein-coupling also leads to inhibition of adenylate cyclase. In addition to endogenous GABA, $GABA_B$ receptors also bind agonists such as baclofen and APPA, and antagonists such as 2-OH-saclofen, phaclofen and SCH50911 (Bonanno and Raiteri, 1993). $GABA_B$ receptor modulators have been used in the treatment of spasticity and neuralgia, and such agents may have therapeutic potential in the treatment of drug abuse, and as anti-depressants, anti-convulsants and enhancers of cognition (Cousins *et al.*, 2002; Bowery and Smart, 2006; Couve *et al.*, 2000).

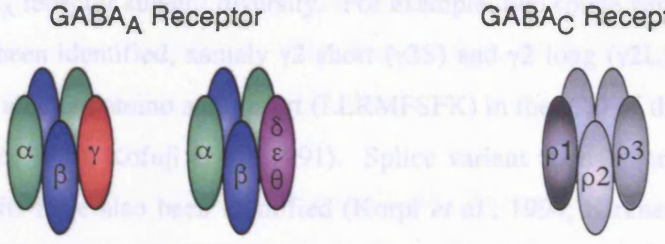
1.3 $GABA_A$ Receptor Structure and Assembly

The ionotropic $GABA_A$ (and $GABA_C$) receptors belong to the superfamily of ligand-gated ion channels, members of which include the nicotinic acetylcholine receptor, the glycine receptor and the 5-hydroxytryptamine type 3 (5-HT-3) receptor (Ortells and Lunt, 1995; Schofield *et al.*, 1987; Unwin, N, 1993; Maricq *et al.*, 1991; Grenningloh *et al.*, 1987). All receptors within this superfamily are hetero-pentameric ion channels assembled with subunits that share a common topology (Ortells and Lunt, 1995; Unwin, N, 1998). Indeed, $GABA_A$ (and $GABA_C$) receptor subunits each have extracellular N- and C-terminal domains, four putative hydrophobic transmembrane (TM) regions (TM1-TM4) and a major intracellular domain (ICD) between TM3 and TM4 (Fig. 1.1A) (Nayeem *et al.*, 1994; Macdonald and Olsen, 1994). The large, extracellular N-terminal region contains a short conserved cysteine loop and putative sites for glycosylation, ligand binding and subunit oligomerisation (Taylor *et al.*, 2000, 1999; Klausberger *et al.*, 2000, 2001a,b;

A.



B.



α1-6, β1-3, γ1-3,
δ, ε, π and θ

ρ1-3

Adapted from Moss and Smart, 2001

Figure 1.1. Schematic representation of the structure of GABA$_A$ receptors. A. GABA$_A$ (and GABA$_C$) receptor subunits contain extracellular N- and C-terminal regions and four hydrophobic transmembrane (TM) domains, numbered 1-4. TM2 (coloured light green) is believed to line the pore of the putative pentameric ion channel complex. The major intracellular domain between TM3 and TM4 is the most divergent region of the subunit. It mediates numerous protein-protein interactions and is subject to post-translational modifications, such as phosphorylation. **B.** Most neuronal GABA$_A$ receptors are believed to comprise α, β and γ subunits in the ratio 2:2:1. In some complexes, the γ subunit is exchanged for a δ, ε or θ subunit. GABA$_C$ receptors are homomeric or heteromeric pentameric assemblies of ρ1-3 subunits. Their subunit stoichiometry is unknown.

Srinivasan *et al.*, 1999; Macdonald and Olsen, 1994; Kittler *et al.*, 2002). TM2 of each subunit is believed to line the channel (Fig. 1.1A). The ICD is the least conserved region of the subunit protein. It mediates numerous protein-protein interactions and is subject to post-translational modifications, such as palmitoylation and phosphorylation (see Section 1.7.2) (Rathenberg *et al.*, 2004; Keller *et al.*, 2004; Moss and Smart, 2001).

There are currently 16 known mammalian $GABA_A$ receptor subunits, which can be arranged into seven classes according to amino acid sequence identity: α1-6, β1-3, γ1-3, δ, ε, π and θ (Fig. 1.1B) (Rabow *et al.*, 1995; Davies *et al.*, 1997; Hedblom and Kirkness, 1997; Bonnert *et al.*, 1999; Moss and Smart, 2001). An additional distinct class of subunits, the ρ1–3 subunits, has also been described. These assemble as homo- or hetero-oligomers to form $GABA_C$ receptors (Fig. 1.1B; Section 1.2) (Bormann, 2000; Mehta and Ticku, 1999). Alternative mRNA splicing generates further $GABA_A$ receptor subunit diversity. For example, two splice variants of the γ2 subunit have been identified, namely γ2 short (γ2S) and γ2 long (γ2L), which differ on account of an eight amino acid insert (LLRMFSFK) in the ICD of the γ2L subunit (Whiting *et al.*, 1990; Kofuji *et al.*, 1991). Splice variant forms of mammalian α6 and β3 subunits have also been identified (Korpi *et al.*, 1994; Kirkness and Fraser, 1993).

$GABA_A$ receptor subunits are differentially expressed throughout the brain in a spatially and temporally controlled manner (Wisden *et al.*, 1992; Laurie *et al.*, 1992a,b; Fritschy and Mohler, 1995; Pirker *et al.*, 2000). For example, cerebellar granule cells are enriched with mRNA of the α6 subunit but do not appear to contain mRNA of the α5 subunit (Laurie *et al.*, 1992a). Furthermore, in the cortex, mRNA of the β3 subunit is markedly expressed prenatally, whereas mRNA of the β2 subunit is mainly expressed postnatally (Laurie *et al.*, 1992b). Individual neurons express a variety of $GABA_A$ receptor subunits, which assemble into various receptor complexes. The large pool of $GABA_A$ receptor subunits provides potential for extensive receptor heterogeneity. However, although the precise subunit composition of native receptors remains unknown, the majority of neuronal receptors are believed

to be composed of α, β and γ subunits in a ratio of 2 to 2 to 1, respectively (Fig. 1.1B) (Tretter *et al.*, 1997; Farrar *et al.*, 1999; Chang *et al.*, 1996). Indeed, the most prevalent receptor complex has been suggested to contain α1, β2 and γ2 subunits (Sieghart and Sperk, 2002; McKernan and Whiting, 1996). In addition, some receptor assemblies are thought to contain a δ, ε or θ subunit in place of the γ subunit (Fig. 1.1B), and form functional high-affinity $GABA_A$ receptors that are insensitive to benzodiazepines (Moss and Smart, 2001; McKernan and Whiting, 1996; Saxena and MacDonald, 1996). Indeed, evidence suggests that the binding site for GABA is located at the interface between the α and β subunits, and the binding site for benzodiazepines at the interface between the α and γ subunits (Baumann *et al.*, 2003; Sigel and Buhr, 1997).

In addition to determining pharmacological properties of $GABA_A$ receptors, subunit composition is also involved in determining channel properties and the clustering and sub-cellular targeting of the receptors, as discussed in the following section (Rudolph *et al.*, 2001; Macdonald and Olsen, 1994; Kittler *et al.*, 2002; Nusser *et al.*, 1996, 1998a; Fritschy *et al.*, 1998; Koulen *et al.*, 1996).

1.4 Membrane Clustering and Targeting of $GABA_A$ Receptors

Subunit composition is believed to be a major determinant of the sub-cellular localisation of $GABA_A$ receptors. For example, in hippocampal pyramidal neurons α2 subunit-containing $GABA_A$ receptors are predominantly localised to the axon initial segment, whereas α5 subunit-containing $GABA_A$ receptors are found at extrasynaptic sites (Nusser *et al.*, 1996; Fritschy *et al.*, 1998; Brunig *et al.*, 2002; Caraiscos *et al.*, 2004). Furthermore, in cerebellar granule cells, γ2 subunit-containing $GABA_A$ receptors are localised mainly at synaptic sites, whereas δ subunit-containing $GABA_A$ receptors are located exclusively at extrasynaptic sites (Nusser *et al.*, 1998a; Brickley *et al.*, 1999; Moss and Smart, 2001).

The synaptic targeting and clustering of $GABA_A$ receptors is a critical determinant of the efficacy of inhibitory synaptic transmission and animal behaviour (Kneussel and Betz, 2000; Crestani *et al.*, 1999; Essrich *et al.*, 1998). However, the mechanisms

involved in the accumulation and clustering of $GABA_A$ receptors remain elusive. The γ2 subunit is believed to be a component of all postsynaptic $GABA_A$ receptors, and analyses of γ2 (-/-) subunit knockout mice have revealed a critical role of this subunit in the postsynaptic localization and clustering of $GABA_A$ receptors containing γ2, α1 and α2 subunits, and the multifunctional tubulin-binding protein, gephyrin (Essrich *et al.*, 1998). Whereas TM4 of the γ2 subunit appears to be necessary and sufficient for the clustering of postsynaptic receptors, both TM4 and the major intracellular domain of this subunit appear to be involved in the accumulation of gephyrin (Alldred *et al.*, 2005).

Gephyrin is essential for the localization of glycine receptors at synaptic sites, and is believed to be involved in the accumulation and clustering of $GABA_A$ receptors at inhibitory synapses (Essrich *et al.*, 1998; Kneussel *et al.*, 1999; Levi *et al.*, 2004; Kneussel and Betz, 2000). This 93-kDa protein has been shown to co-localise with $GABA_A$ receptors in various brain regions, though a direct interaction between gephyrin and $GABA_A$ receptors has not yet been described (Essrich *et al.*, 1998; Kneussel and Betz, 2000). Analysis of gephyrin (-/-) knockout mice has revealed that gephyrin is involved in the clustering of most $GABA_A$ receptor γ2 and α2 subunits in spinal cord and cultured hippocampal neurons (Kneussel *et al.*, 1999; Levi *et al.*, 2004). Furthermore, antisense-mediated knockdown of gephyrin has revealed that this protein plays a major role in the clustering of $GABA_A$ receptor γ2 and α2 subunits in cultured cortical neurons (Essrich *et al.*, 1998).

Interestingly, the presynaptic transmembrane protein, neurexin, has been shown to trigger postsynaptic clustering of $GABA_A$ receptors and gephyrin, an effect that is believed to be mediated via the postsynaptic transmembrane counterpart protein, neuroligin-2 (Graf *et al.*, 2004). However, although neuroligin-2 has been localised to GABAergic synapses, it does not appear to interact directly with either $GABA_A$ receptors or gephyrin (Graf *et al.*, 2004). This suggests that other $GABA_A$ receptor-associated proteins are involved in the aggregation of these receptors. Subunit-specific differences in the mechanism of $GABA_A$ receptor clustering have also been proposed and further support this view. For example, the clustering of $GABA_A$

receptor α1 and α5 subunits in the spinal cord is believed to be independent of gephyrin, and therefore involve other, possibly unidentified, proteins (Kneussel *et al.*, 2001). Interestingly, a recent study has demonstrated that the activated actin-binding protein, radixin, is a direct binding-partner of the $GABA_A$ receptor α5 subunit, and is involved in the clustering of these subunits in cultured hippocampal neurons (Loebrich *et al.*, 2006). The protein rapsyn has also been implicated in the clustering of $GABA_A$ receptors containing α1, β1 and γ2 subunits in QT-6 cells (Yang *et al.*, 1997).

1.5 Stability and Dynamics of $GABA_A$ Receptors at the Cell Surface

The number of $GABA_A$ receptors localised at postsynaptic sites is a major determinant of inhibitory synaptic strength (Nusser *et al.*, 1997, 1998b; Otis *et al.*, 1994) (see Section 5.1). The level of expression of postsynaptic $GABA_A$ receptors therefore has significant consequences for neuronal excitability. $GABA_A$ receptors are believed to cycle continuously between the plasma membrane and intracellular compartments (Kittler *et al.*, 2000a, 2004a; Connolly *et al.*, 1999a,b). The relative rates of receptor insertion and removal into or from the neuronal membrane are therefore thought to play a major role in determining the number of $GABA_A$ receptors at synaptic sites. The lateral diffusion of $GABA_A$ receptors in the plane of the membrane has also been suggested to control the movement of receptors into and out of synaptic sites (Thomas *et al.*, 2005; Jacob *et al.*, 2005).

$GABA_A$ receptors can be delivered to the plasma membrane as newly assembled complexes via a *de novo* secretory pathway, or reinserted into the neuronal membrane following internalisation (these processes are discussed in Sections 1.6 and 5.1). However, it is not yet known whether $GABA_A$ receptors are inserted directly into synaptic sites, or whether they first incorporate into extrasynaptic membrane regions and subsequently accumulate at postsynaptic specialisations.

1.6 Trafficking of $GABA_A$ Receptors to the Cell Surface

The oligomerisation of $GABA_A$ receptor subunits into channel complexes is believed to occur in the endoplasmic reticulum, and evidence suggests that this assembly

process is involved in regulating the trafficking of channel complexes to the cell surface (reviewed in Kittler *et al.*, 2002). In addition, a number of cytoplasmic proteins have been identified that appear to be involved in delivering $GABA_A$ receptors to the neuronal membrane.

The 17-kDa, $GABA_A$ receptor-associated protein, GABARAP, belongs to a family of proteins involved in membrane transport, members of which include the transport factor GATE-16 and light-chain 3 of MAP1A and MAP1B (Sagiv *et al.*, 2000; Kabeya *et al.*, 2000; Mann and Hammarback, 1994; Kittler *et al.*, 2002). Several lines of evidence suggest that GABARAP is involved in the trafficking of $GABA_A$ receptors. Biochemical and immunofluorescence experiments have shown that GABARAP coimmunoprecipitates with $GABA_A$ receptors from brain, and co-localises with $GABA_A$ receptors in cultured cortical neurons (Wang *et al.*, 1999; Leil *et al.*, 2004). *In vitro* binding studies have further revealed that GABARAP selectively interacts with the major intracellular domain of the $GABA_A$ receptor γ subunit (Nymann-Anderson *et al.*, 2002; Wang *et al.*, 1999). Interestingly, this interaction appears to be involved in, though is not essential for, the trafficking of $GABA_A$ receptors to the neuronal surface (Leil *et al.*, 2004; O'Sullivan *et al.*, 2005). Localisation studies have revealed that GABARAP is associated predominantly with intracellular structures, such as the Golgi apparatus and a sub-synaptic tubulo-vesicular compartment, which is consistent with a role of this protein in $GABA_A$ receptor transport (Kittler *et al.*, 2001). Furthermore, GABARAP has been shown to interact with NSF (Kittler *et al.*, 2001), a direct binding-partner of $GABA_A$ receptors (Goto *et al.*, 2005) that is involved in membrane trafficking processes. GABARAP and NSF have also been shown to co-localise on intracellular membranes in cultured neurons (Kittler *et al.*, 2001). The interaction between GABARAP and tubulin has been proposed to form a link between $GABA_A$ receptors and the cytoskeleton that may be involved in the trafficking and clustering of $GABA_A$ receptors (Wang and Olsen, 2000; Wang *et al.*, 1999; Coyle *et al.*, 2002; Chen *et al.*, 2000). Indeed, GABARAP has been shown to promote the clustering of $GABA_A$ receptors that contain the $\gamma 2$ subunit in QT-6 quail fibroblasts (Chen *et al.*, 2000). GABARAP has also been shown to interact with the synaptic proteins gephyrin (Kneussel *et al.*,

2000) and GRIP1 (glutamate receptor interacting protein type 1) (Kittler *et al.*, 2004b). However, the association with gephyrin does not play a role in the anchoring of synaptic $GABA_A$ receptors (Kneussel *et al.*, 2000), and gephyrin and GABARAP do not co-localise significantly in cultured neurons (Kittler *et al.*, 2001). Indeed, ultrastructural studies suggest that GABARAP is not a major component of inhibitory synapses (Kittler *et al.*, 2001). GABARAP may therefore induce $GABA_A$ receptor clustering indirectly by enhancing the movement of these receptors to the neuronal surface. Notably, an *in vitro* interaction between the GABARAP-like protein, GEC1, and both $GABA_A$ receptors and tubulin has also been described (Mansuy *et al.*, 2004). However, the role of this association in the trafficking of $GABA_A$ receptors remains to be determined.

The phospholipase C-related catalytically inactive protein type 1 (PRIP-1; also known as p130) is believed to be involved in regulating the function of $GABA_A$ receptors. Indeed, it has been suggested that PRIP-1 may be involved in the phosphorylation-dependent regulation and trafficking of these receptors. PRIP-1 coimmunoprecipitates with $GABA_A$ receptor $\beta 3$ subunits from brain, and binds directly and selectively to the major intracellular domains of the receptor β subunits (Terunuma *et al.*, 2004). PRIP-1 also binds the serine/threonine phosphatase $PP1\alpha$, and is believed to target this enzyme to the $GABA_A$ receptor (Terunuma *et al.*, 2004; Yoshimura *et al.*, 2001; Uji *et al.*, 2002). This anchoring process has been proposed to play a role in regulating the phosphorylation state and activity of $GABA_A$ receptors, and is discussed further in Section 1.7.2.2.1 (Terunuma *et al.*, 2004; Yoshimura *et al.*, 2001). PRIP-1 (and its homologue PRIP-2) has also been shown to bind GABARAP (Kanematsu *et al.*, 2002; Yoshimura *et al.*, 2001; Terunuma *et al.*, 2004; Uji *et al.*, 2002). This interaction may regulate the effect of GABARAP on the trafficking and clustering of $\gamma 2$ subunit-containing $GABA_A$ receptors, as PRIP-1 and the receptor $\gamma 2$ subunit have been shown to compete for the interaction with GABARAP (Kanematsu *et al.*, 2002; Uji *et al.*, 2002). Indeed, the behaviour and $GABA_A$ receptor pharmacology of PRIP-1 (-/-) knockout mice is consistent with an altered function of $\gamma 2$ subunit-containing $GABA_A$ receptors (Kanematsu *et al.*, 2002).

The ubiquitin-like protein Plic-1 (protein that links integrin-associated protein with the cytoskeleton type-1) has also been implicated in regulating $GABA_A$ receptor trafficking. Plic-1 is believed to be involved in stabilising proteins by interfering with ubiquitin-mediated proteolysis (Kleijnen *et al.*, 2000; Mah *et al.*, 2000). This 67-kDa protein has been shown to bind the major intracellular domains of $GABA_A$ receptor α and β subunits, and these interactions are believed to increase the half-life of intracellular $GABA_A$ receptors, and facilitate their delivery to the neuronal surface (Bedford *et al.*, 2001). This putative role is consistent with localisation studies, which have detected Plic-1 at a number of intracellular compartments, including the Golgi apparatus and sub-synaptic cisternae, in addition to GABAergic synapses (Bedford *et al.*, 2001). These findings further suggest that Plic-1 may facilitate the insertion of both internalised and newly synthesised receptors into the plasma membrane.

The huntingtin-associated protein type-1 (HAP-1) is also believed to play a role in regulating the function of $GABA_A$ receptors. HAP-1 has been shown to coimmunoprecipitate with β subunit-containing $GABA_A$ receptors from brain, and to bind selectively to the major intracellular domains of the receptor β subunits, *in vitro* (Kittler *et al.*, 2004a). HAP-1 has also been shown to co-localise with $\gamma 2$ subunit-containing $GABA_A$ receptors in cultured hippocampal neurons (Kittler *et al.*, 2004a). The interaction of HAP-1 with the $GABA_A$ receptor is believed to play a role in the post-endocytic sorting and trafficking of $GABA_A$ receptors, as overexpression of HAP-1 in cultured neurons has been shown to inhibit the degradation of internalised $GABA_A$ receptors, and promote receptor recycling to the plasma membrane (Kittler *et al.*, 2004a). It has also been shown to increase the number of synaptic $GABA_A$ receptors, and to potentiate the mean amplitude of miniature inhibitory post-synaptic currents (mIPSCs) (Kittler *et al.*, 2004a). Previous studies have implicated HAP-1 in membrane transport and post-endocytic protein sorting (Gutekunst *et al.*, 1998; Martin *et al.*, 1999; Li *et al.*, 2002; Engelender *et al.*, 1997). Indeed, this protein has been shown to bind a number of proteins involved in membrane transport, such as the $p150^{glued}$ subunit of dynactin (Engelender *et al.*, 1997) and the light-chain subunit of the microtubule-based kinesin motor complex (McGuire *et al.*, 2006). Interestingly,

GRIP1, which co-localises with $GABA_A$ receptors in cultured neurons (Kittler *et al.*, 2004b; Charych *et al.*, 2004a), and GRIF-1 ($GABA_A$ receptor interacting factor type-1), which binds β2 subunit-containing $GABA_A$ receptors (Beck *et al.*, 2002), have also been shown to interact with kinesins (Setou *et al.*, 2002; Brickley *et al.*, 2005; Pozo and Stephenson, 2006). A number of proteins may therefore be involved in $GABA_A$ receptor trafficking by linking receptor complexes to microtubule motors. However, it remains to be determined whether $GABA_A$ receptors bind directly to kinesins and GRIP1.

Another protein that is believed to play a role in regulating $GABA_A$ receptor function is BIG2 (brefeldin A-inhibited guanine nucleotide-exchange protein type 2), which has been shown to activate small GTPases of the ADP-ribosylation factor (ARF) family, and play an important role in vesicular trafficking (Morinaga *et al.*, 1997; Welsh *et al.*, 1994; Jones *et al.*, 2005). The interaction of BIG2 with β subunit-containing $GABA_A$ receptors has been implicated in facilitating the trafficking of $GABA_A$ receptors to the cell surface, a role that is consistent with localisation studies, which have detected BIG2 in various intracellular compartments, including the trans-Golgi network, recycling endosomes and inhibitory synapses (Charych *et al.*, 2004b; Shin *et al.*, 2004).

It is now believed that $GABA_A$ receptors are subject to regulation by palmitoylation (or thioacylation) (Keller *et al.*, 2004; Rathenberg *et al.*, 2004), a reversible post-translational modification that is known to regulate the localisation of numerous proteins, and which involves the addition of the 16-carbon fatty acid, palmitate, to cysteine residues (Huang and El-Husseini, 2005). Recent studies have identified an association between $GABA_A$ receptors and the palmitoyl acyl transferase, GODZ (Golgi-specific DHHC zinc finger protein) (Keller *et al.*, 2004; Uemura *et al.*, 2002). GODZ binds selectively to a region of the receptor γ subunit that contains a number of conserved cysteine residues (Keller *et al.*, 2004), and is believed to be involved in palmitoylation of these sites (Keller *et al.*, 2004; Rathenberg *et al.*, 2004). Palmitoylation of $GABA_A$ receptors has been shown to enhance both receptor clustering and cell-surface expression (Rathenberg *et al.*, 2004). These effects may

result from an enhancement in the trafficking of receptors to the neuronal surface, which would be consistent with the enrichment of GODZ in the Golgi apparatus (Uemura *et al.*, 2002), and a reduction in the internalisation of receptors from the cell surface. Indeed, this would be consistent with GODZ-mediated palmitoylation of AMPA receptors, which regulates receptor levels in the Golgi apparatus and receptor internalisation (Hayashi *et al.*, 2005).

Protein kinases have also been implicated in the cell-surface trafficking of $GABA_A$ receptors. For example, activation of PKC has been shown to reduce the level of expression of recombinant γ2 subunit-containing $GABA_A$ receptors on the cell surface of HEK-293 cells (Connolly *et al.*, 1999a). This effect is believed to result from inhibition of receptor recycling to the plasma membrane following internalisation, and has been shown to be independent of the PKC phosphorylation sites (Connolly *et al.*, 1999a). Similar results have also been seen in *Xenopus* oocytes (Chapell *et al.*, 1998). The role of direct receptor phosphorylation in the trafficking of $GABA_A$ receptors is discussed in Section 1.7.3 and in Chapter 5.

1.7 Phosphorylation-Dependent Functional Modulation of $GABA_A$ Receptors

1.7.1 Phosphorylation-Dependent Modification of Proteins is a Key Component of Intracellular Signalling Networks and Modulates Various Neuronal Processes

Phosphorylation and dephosphorylation by protein kinases and phosphatases is fundamental to the activity of cellular signalling networks. Phosphorylation is one of the most well-studied and ubiquitous post-translational modifications (PTMs) of protein structure. Like other PTMs, such as palmitoylation and ubiquitination, phosphorylation regulates protein activity.

Protein phosphorylation involves the transfer of the terminal (γ) phosphoryl group of a magnesium-ATP complex to an intracellular protein at a serine, threonine or tyrosine residue. This occurs in a reaction catalysed by either a serine/threonine- or tyrosine-specific protein kinase. Phosphorylation is a covalent modification and can be reversed by hydrolysis of the phosphoryl group in a reaction catalysed by a protein

phosphatase. Phosphorylation reactions are characterised by distinct kinetics, and can trigger transient or long-lasting downstream events. The addition of a phosphoryl moiety to a protein induces conformational change due to alterations in properties such as charge and hydrogen-bonding capacity. This structural modification can alter protein interactions and the catalytic activity of enzymes, as well as ion channel and receptor function.

The human genome encodes for 518 protein kinases and an estimated 150 protein phosphatases (Manning *et al.*, 2002; Papin *et al.*, 2005). This diversity is increased further by alternative splicing of the phosphoregulator genes, which produces mRNA transcripts for ~1,295 kinase and ~375 phosphatase isoforms (Papin *et al.*, 2005). The complexity of phosphorylation-dependent signalling networks is believed to underlie the remarkable specificity of phosphorylation-dependent signalling responses to various extracellular stimuli, and is increased further by the combinatorial assembly of kinases, phosphatases and other protein complexes (Papin *et al.*, 2005). Protein kinases and phosphatases often recognise specific binding and phosphorylation motifs, which are present in a number of different proteins. Consequently, individual cells contain large repertoires of potential kinase binding-partners and substrates. As well as increasing the potential for combinatorial interactions, this forms a basis for signal amplification. Indeed, phosphorylation-dependent signalling pathways can be particularly extensive if any of the target substrates proceed to modulate a further set of proteins. However, tight spatio-temporal control of the expression, alternative-splicing, sub-cellular targeting and stability of signalling proteins plays a critical role in limiting combinatorial protein interactions and in restricting the potential complexity of phosphorylation-dependent signalling (Papin *et al.*, 2005).

One-third of intracellular proteins are estimated to be phosphoproteins (Johnson and Hunter, 2005). Many of these substrates can be modulated by more than one protein kinase (or PTM or other signalling component). Although this can lead to convergence of signalling pathways, it can also increase the number of functional states of a protein, particularly if the upstream modulators have multiple and distinct

sites of phosphorylation. This can enable a phosphoprotein to exert specific functions in a regulated manner in response to various external stimuli.

The profound importance of phosphorylation-dependent modulation and signal integration in the control of neuronal signalling and animal behaviour is exemplified in a series of experiments examining the dopamine and cyclic AMP-regulated phosphoprotein of 32-kDa (DARRP-32), which can be regarded as a type of neuronal intracellular signalling hub. DARRP-32 has multiple functional states determined by the phosphorylation state of four distinct sites (Greengard *et al.*, 2001; Svenningsson *et al.*, 2003). Phosphorylation of T34 by PKA converts DARRP-32 into a potent inhibitor of PP1 (Hemmings *et al.*, 1984), and this effect can be enhanced by phosphorylation of S97 (Girault *et al.*, 1989). In addition to this, *in vitro* phosphorylation of S130 by casein kinase-1 has also been shown to prevent dephosphorylation of phospho-T34 by PP2B (Desdouits *et al.*, 1995). Phosphorylation of T75 by cyclin-dependent kinase 5 (Cdk5) converts DARRP-32 into an inhibitor of PKA, and causes activation of PP1 (Bibb *et al.*, 1999). Collectively, these studies suggest that phosphorylation events are important determinants of downstream PP1 and PKA signalling (Greengard *et al.*, 1999). Interestingly, a further study by Greengard and colleagues showed that treating mice with psychotomimetics, which disrupt glutamatergic, serotonergic or dopaminergic neurotransmission, alters the phosphorylation state of DARRP-32 at three of the above-mentioned sites in a manner predicted to cause synergistic blockade of PP1 function (Svenningsson *et al.*, 2003). The authors showed that some of the behavioural effects of these psychotomimetics are dependent upon DARRP-32 phosphorylation by using DARRP-32 knockout mice and phosphomutant mice containing alanine point mutations at various DARRP-32 phosphorylation sites (Svenningsson *et al.*, 2003).

The co-ordinated activity of protein kinases and phosphatases plays a pivotal role in controlling many neuronal processes, including neurotransmission and synaptic plasticity (Turner *et al.*, 1999; Swope *et al.*, 1999; Levitan, 1999, 1994). Indeed, the phosphorylation of ligand-gated ion channels can regulate several receptor properties,

such as channel activity and receptor trafficking, and have a significant impact on the efficacy of synaptic transmission and neuronal excitability (Wang *et al.*, 2005; Luscher and Keller, 2004; Turner *et al.*, 1999; Levitan, 1999). It is therefore essential to elucidate the precise mechanisms by which protein kinases and phosphatases regulate the phosphorylation state and activity of ligand-gated ion channels.

1.7.2 $GABA_A$ Receptors undergo Phosphorylation-Dependent Modification

$GABA_A$ receptors are well-established phosphoproteins. Evidence suggests that the phosphorylation state of these receptors is determined by the activity of various serine/threonine and tyrosine protein kinases and phosphatases (Moss and Smart, 2001). A number of these enzymes have been shown to phosphorylate/dephosphorylate specific residues located within the major intracellular domain of particular receptor subunits in neurons, heterologous cell lines and *in vitro* (Moss and Smart, 2001; Kittler and Moss, 2003; Table 1.1). The targeting of individual protein kinases and phosphatases to $GABA_A$ receptors is believed to ensure stoichiometric phosphorylation of the receptor subunits. Indeed, emerging evidence suggests that $GABA_A$ receptors are components of dynamic protein scaffolds containing various phosphomodulators and other phosphorylation-dependent signalling components (see Fig. 3.9).

In this section, I will discuss the known roles of protein kinases and phosphatases in phosphorylation-dependent modification of $GABA_A$ receptors. I will discuss the known physical interactions between $GABA_A$ receptors and these signalling enzymes, the known sites of phosphorylation and the effects of this modification on $GABA_A$ receptor function. Finally, I shall discuss some known roles of phosphorylation-dependent signalling in $GABA_A$ receptor cross-talk in neurons.

1.7.2.1 The Role of Protein Kinases

1.7.2.1.1 PKC and the RACK-1 Anchoring Protein

PKC has been extensively investigated as a $GABA_A$ receptor kinase. *In vitro* biochemical studies using purified, bacterially expressed GST-fusion proteins of the

$GABA_A$ Receptor Subunit	Phosphorylation Site	Protein Kinase		
		In Vitro	Heterologous Cell-Lines	Primary Neurons
α1	T337	GAPDH		
	S416	GAPDH		
β1	S384	CaMKII		
	S409	PKC, PKA, CaMKII, PKG	PKC, PKA	
	Y370		vSRC	
	Y372		vSRC	
β2	S410	PKC, PKA, Akt, CaMKII, PKG	PKC, Akt	Akt
β3	S383	CaMKII		
	S408	PKC	PKC, PKA	PKC, PKA
	S409	PKC, PKA, CaMKII, PKG	PKC, PKA	PKC, PKA
γ2	S327	PKC		
	S343 (in γ2L only)	PKC, CaMKII		
	S348	CaMKII		
	T350	CaMKII		
	Y365	cSrc	vSRC	
	Y367	cSrc	vSRC	

(Adapted from Brandon et al., 2002)

Table 1.1. Sites of phosphorylation in $GABA_A$ receptor subunits. Phosphorylation sites were identified from *in vitro* studies using purified GST-fusion proteins of the major intracellular domains of $GABA_A$ receptor subunits, and from studies of recombinant receptors expressed in heterologous cell lines, or native receptor complexes in primary neuronal cultures. Protein kinases involved in phosphorylation of individual sites in a particular system are indicated where known. See text for references.

major intracellular domains of $GABA_A$ receptor subunits have revealed that this serine/threonine protein kinase selectively phosphorylates the β and γ receptor subunits, but not the α1 subunit (Moss *et al.*, 1992a; McDonald and Moss, 1997). Further analysis utilising site-directed mutagenesis showed that the β subunits are each phosphorylated by PKC at a conserved serine residue, namely S409 of the β1 subunit (Moss *et al.*, 1992a), S410 of the β2 subunit, and S409 of the β3 subunit (McDonald and Moss, 1997) (Table 1.1). An additional site of phosphorylation, S408, was also identified in the β3 subunit (McDonald and Moss, 1997) (Table 1.1). These studies also showed that the γ2S and γ2L subunits are phosphorylated by PKC at S327 (Moss *et al.*, 1992a), and that the γ2L subunit contains a second site at S343, within the alternatively spliced eight amino acid insertion (Moss *et al.*, 1992a; Machu *et al.*, 1993; Whiting *et al.*, 1990) (Table 1.1).

PKC has been shown to phosphorylate the full-length β subunits of purified $GABA_A$ receptors *in vitro* (Browning *et al.*, 1990, 1993). Pre-labelling experiments with [^{32}P]-orthophosphate have subsequently been used to examine the phosphorylation of recombinant and neuronal $GABA_A$ receptors by PKC *in situ*. These have shown PKC-induced phosphorylation of each of the $GABA_A$ receptor β subunits, when expressed with an α1 subunit in HEK293 cells (Krishek *et al.*, 1994; McDonald *et al.*, 1998; Brandon *et al.*, 2000) (Table 1.1). The sites of phosphorylation in the β1 and β2 subunits were identical to those identified *in vitro*, namely S409 and S410, respectively (Krishek *et al.*, 1994; McDonald *et al.*, 1998). Likewise, the β3 subunit is believed to be phosphorylated at S408 and S409 (Brandon *et al.*, 2000). Additional studies utilising pre-labelling assays or a phosphorylation state-specific antibody that recognises the $GABA_A$ receptor β3 subunit phosphorylated at S408 and S409 (anti-β3-p408/409) (Jovanovic *et al.*, 2004) were used to investigate PKC activity in a neuronal system. These analyses revealed PKC-dependent phosphorylation of the $GABA_A$ receptor β3 subunit at S408 and S409 in primary cultures of neurons under basal conditions (Brandon *et al.*, 2000, 2002; Jovanovic *et al.*, 2004). They also showed that the basal level of β3 subunit phosphorylation is increased following activation of PKC with a phorbol ester, muscarine or BDNF (Brandon *et al.*, 2000,

2002, 2003; Jovanovic *et al.*, 2004), and decreased following inhibition of PKC with calphostin C (Brandon *et al.*, 2000).

PKC interacts directly with $GABA_A$ receptor β subunits at a region that encompasses the site of PKC phosphorylation. Affinity-purification assays using GST-fusion proteins of the major intracellular domains of $GABA_A$ receptor subunits have demonstrated that PKC binds selectively to the $GABA_A$ receptor β1-3 subunits, but not the α1 or γ2 subunits, and that the associated kinase phosphorylates S409 of the β1 subunit, and S408 and S409 of the β3 subunit (Brandon *et al.*, 1999, 2002, 2003). Further analysis revealed that the intracellular domain of the β1 subunit interacts directly with the α and βII isoforms of PKC (PKC-α and PKC-βII) (Brandon *et al.*, 1999), that the intracellular domain of the β2 subunit interacts with PKC-α (Brandon *et al.*, 2003), and that the intracellular domain of the β3 subunit associates with PKC-α and PKC-βII (Brandon *et al.*, 1999). Mapping studies also found that PKC-βII binds to a site located between residues 405 and 415 of the β1 subunit major intracellular domain, a region which includes the sole site of phosphorylation, S409 (Brandon *et al.*, 2002). Both the binding and phosphorylation sites are conserved between the β subunit isoforms.

The level of PKC binding to $GABA_A$ receptors is enhanced following activation of the kinase. Coimmunoprecipitation assays have shown that an increase in the association of PKC with β1/3 subunit-containing $GABA_A$ receptors is caused by treatment of cultured cortical neurons with phorbol esters (Brandon *et al.*, 1999). A similar study has also shown that treatment of cultured cortical neurons with BDNF triggers activation of PKC, and induces temporary docking of the kinase to $GABA_A$ receptors that contain β3 subunits (Jovanovic *et al.*, 2004). The transient nature of the interaction between $GABA_A$ receptors and PKC in primary neurons has been attributed in part to the kinase having differential binding-affinities for phosphorylated and dephosphorylated forms of the β3 subunit. This view is based on findings from *in vitro* binding studies and experiments using a phosphorylation-state specific anti-β3-p408/409 antibody. These studies demonstrated that although the interaction between PKC and the major intracellular domain of the β1 subunit is not

abolished by a serine-to-alanine point mutation of the S409 phosphorylation site, the extent of binding is reduced when this residue is phosphorylated by PKA (Brandon *et al.*, 1999, 2002). Similarly, binding of PKC to the major intracellular domain of the β3 subunit is not dependent upon the PKC phosphorylation sites, S408 and S409, but is reduced by phosphorylation of the S409 residue by PKA (Brandon *et al.*, 1999; Jovanovic *et al.*, 2004). It is not yet known whether the phospho-dependent interaction between PKC and the $GABA_A$ receptor β subunits results from the kinase having a reduced binding-affinity or an increased rate of dissociation for the phosphorylated form of the β subunit, or whether there are additional contributing factors. Nevertheless, this regulation provides a potential mechanism for PKC to negatively modulate its own binding and phosphorylation, *in vivo*. Indeed, activity- and phosphorylation-dependent binding is believed to contribute to the transient increase in PKC-dependent phosphorylation of the $GABA_A$ receptor β3 subunit at S408 and S409 that is observed following treatment of cultured cortical neurons with muscarine, BDNF or phorbol esters (Brandon *et al.*, 1999, 2000, 2002; Jovanovic *et al.*, 2004).

The anchoring protein RACK-1 regulates $GABA_A$ receptors by facilitating PKC-dependent phosphorylation (Brandon *et al.*, 1999, 2002; Feng *et al.*, 2001). Like PKC-βII, RACK-1 immunoprecipitates with $GABA_A$ receptors containing β1/β3 subunits from HEK-293 cells, brain and cultured cortical neurons that have been treated with phorbol esters (Brandon *et al.*, 1999, 2002). In cultured cortical neurons, activation of Trk B neurotrophin receptors with BDNF has been shown to induce transient recruitment of RACK-1, in addition to PKC, to $GABA_A$ receptors containing the β3 subunit (Jovanovic *et al.*, 2004). Although RACK-1 binds directly to PKC-α and PKC-βII (Brandon *et al.*, 1999; Jaken and Parker, 2000) (and may therefore bind indirectly to $GABA_A$ receptors), a direct interaction between RACK-1 and the major intracellular domains of $GABA_A$ receptor α, β1 and β3 subunits has been demonstrated (Brandon *et al.*, 1999, 2002). RACK-1 binds directly to the major intracellular domain of the β1 subunit between residues 395 and 404 (Brandon *et al.*, 2002). This binding region lies immediately upstream of the binding site for PKC-βII and is conserved between the β subunit isoforms (Brandon *et al.*, 2002). Although

their binding sites are within close proximity of one another, PKC-βII and RACK-1 can bind independently to $GABA_A$ receptor β subunits (Brandon *et al.*, 1999, 2002). Indeed, RACK-1 has been shown to bind a fragment of the intracellular domain of the β1 subunit that does not contain the PKC-βII-binding region (Brandon *et al.*, 2002). Furthermore, binding of RACK-1 to the intracellular domain of the β1 subunit does not require S409, and is not regulated by phosphorylation of this residue (Brandon *et al.*, 2002). A peptide mimicking the RACK-1-binding sequence in the β1 subunit has been shown to block the interaction of RACK-1, but not PKC, with the receptor β1 subunit (Brandon *et al.*, 2002). This peptide has also been shown to reduce PKC-dependent phosphorylation and functional modulation of $GABA_A$ receptors that contain a β1 subunit in HEK-293 cells and superior cervical ganglion neurons (Brandon *et al.*, 2002). RACK-1 is therefore believed to facilitate PKC-dependent phosphorylation and functional modulation of $GABA_A$ receptors by enhancing the activity of receptor-associated PKC, rather than by targeting activated PKC isoforms to the receptor (Brandon *et al.*, 2002).

1.7.2.1.2 PKA and AKAP Scaffold Proteins

The serine/threonine kinase, PKA, has also been identified as a $GABA_A$ receptor kinase. *In vitro* studies using purified, bacterially expressed GST-fusion proteins of the major intracellular domains of $GABA_A$ receptor subunits have revealed that this kinase selectively phosphorylates the receptor β subunits, but not the α1 or γ2 subunits (Moss *et al.*, 1992a; McDonald and Moss, 1997) (Table 1.1). Further studies using site-directed mutagenesis demonstrated that PKA phosphorylates each of the β subunits at the same conserved serine residue as PKC, namely S409 of the β1 and β3 subunits (Moss *et al.*, 1992a; McDonald and Moss, 1997) and S410 of the β2 subunit (McDonald and Moss, 1997) (Table 1.1).

Purified $GABA_A$ receptors are also phosphorylated by PKA, *in vitro* (Kirkness *et al.*, 1989; Browning *et al.*, 1990,1993; Tehrani and Barnes, 1994). However, *in situ* analyses of recombinant $GABA_A$ receptors expressed in HEK-293 cells have revealed a number of differences between the phosphorylation of $GABA_A$ receptor subunits *in vitro* and *in situ*. For example, PKA has been shown to phosphorylate the receptor β1

subunit at S409 (Moss *et al.*, 1992b), which is in agreement with the *in vitro* findings (see above; Table 1.1). However, in contrast to the *in vitro* findings, PKA-dependent phosphorylation of the β3 subunit occurs at S408 and S409 (rather than S409 alone). In this system, phosphorylation also occurs at an unidentified serine residue; and activation of PKA does not lead to phosphorylation of the β2 subunit (McDonald *et al.*, 1998; Table 1.1). In hippocampal slices, activation of PKA with forskolin has been shown to trigger phosphorylation of the $GABA_A$ receptor β2/3 subunits, and experiments using a phosphorylation-state specific anti-β3-pS408/S409 subunit antibody showed that phosphorylation of the β3 subunit occurs at S408/S409 (Terunuma *et al.*, 2004). Activation of PKA also leads to phosphorylation of the β3 subunit at S408/4S09 in primary cultures of striatal neurons (Brandon *et al.*, 2003), and in cultured cortical neurons when endogenous PKC activity is inhibited (Brandon *et al.*, 2000; Table 1.1). These studies collectively suggest that a number of factors are involved in determining the sites of PKA-dependent phosphorylation of $GABA_A$ receptors in cells.

The A-kinase anchoring proteins (AKAPs), AKAP79/150 and Yotiao, are fundamental to PKA-mediated phosphorylation and functional modulation of $GABA_A$ receptors (Brandon *et al.*, 2003; Wang *et al.*, 2002; Cai *et al.*, 2002). These scaffolding proteins interact directly with the regulatory RII subunit of PKA to target the kinase to its substrates (Colledge and Scott, 1999). Indeed, AKAPs are believed to facilitate PKA-dependent phosphorylation of the $GABA_A$ receptor β1 and β3 subunits in cells by targeting the kinase to the major intracellular domains of these subunits (Brandon *et al.*, 2003; McDonald *et al.*, 1998). Affinity-purification assays have demonstrated that native AKAP79/150 interacts selectively with the major intracellular domains of the $GABA_A$ receptor β1 and β3 subunits, but not the β2, α1-3, γ2 or δ subunits, and that the interaction of recombinant AKAP79 with the receptor β1 and β3 subunits is direct (Brandon *et al.*, 2003). Additional studies have also shown that the association of the catalytic subunit of PKA with the receptor β1 and β3 subunits is indirect (Brandon *et al.*, 2003). Although the β1 and β3 receptor subunits may associate with the catalytic subunit of PKA via a direct interaction with the regulatory RII subunit, the binding of PKA to AKAP79 has been shown to be

critical for PKA-mediated phosphorylation of the $GABA_A$ receptor β3 subunit at S408/S409 in heterologous cells (Brandon *et al.*, 2003).

AKAPs have also been shown to interact directly with PKC and PP2B, in addition to PKA (Klauck *et al.*, 1996; Colledge and Scott, 1999). However, the targeting of PKC and PP2B to $GABA_A$ receptors by AKAPs remains to be investigated (see Section 1.7.2.2.3).

1.7.2.1.3 Akt

In addition to PKA and PKC, $GABA_A$ receptors are also substrates of the serine/threonine kinase, Akt, or PKB. Akt selectively phosphorylates the major intracellular domain of the $GABA_A$ receptor β2 subunit, but not the α1 or γ2 subunit, *in vitro* (Wang *et al.*, 2003b) (Table 1.1). Phosphorylation of the β2 subunit occurs at the conserved serine residue, S410, which is also the *in vitro* phosphorylation site of PKA and PKC (Wang *et al.*, 2003b; McDonald and Moss, 1997) (Table 1.1). Activation of Akt has been shown to trigger phosphorylation of the β2 subunit at S410 in HEK293 cells expressing recombinant $GABA_A$ receptors (Wang *et al.*, 2003b) (Table 1.1). Furthermore, endogenous Akt has been implicated in the insulin-induced phosphorylation of native $GABA_A$ receptor β2 subunits at S410 in primary cultures of neurons (Wang *et al.*, 2003b) (Table 1.1). Immunofluorescence studies have shown that insulin induces co-localisation of activated Akt and $GABA_A$ receptors containing the β2 subunit in the distal dendrites of cultured hippocampal neurons (Wang *et al.*, 2003b). However, a direct physical interaction between Akt and the neuronal $GABA_A$ receptor substrate remains to be determined.

1.7.2.1.4 GAPDH

A physical and functional interaction between $GABA_A$ receptors and the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), has now been reported. This serine/threonine protein kinase directly phosphorylates the major intracellular domain of the $GABA_A$ receptor α1 subunit at T337 and S416, *in vitro* (Laschet *et al.*, 2004) (Table 1.1). Furthermore, GAPDH is believed to be the α1-subunit-specific kinase that is associated with purified preparations of $GABA_A$

receptors from bovine cerebral cortex (Sweetnam *et al.*, 1988; Bureau and Laschet, 1995; Laschet *et al.*, 2004). Indeed, in addition to co-purifying with $GABA_A$ receptors from bovine cortex, GAPDH co-localises with $GABA_A$ receptor $\alpha 1$ subunits at the neuronal surface of cultured hippocampal neurons (Laschet *et al.*, 2004).

1.7.2.1.5 CaMKII

In vitro studies using purified GST-fusion proteins of the major intracellular domains of $GABA_A$ receptor subunits have revealed that the serine/threonine kinase, CaMKII, phosphorylates the receptor β and $\gamma 2$ subunits (McDonald and Moss, 1994, 1997). Further investigations using site-directed mutagenesis revealed that the $\beta 1$ subunit is phosphorylated on S384 and S409 (McDonald and Moss, 1994), the $\beta 2$ subunit on S410, and the $\beta 3$ subunit on S383 and S409 (McDonald and Moss, 1997; Table 1.1). Interestingly, whereas the S409/410 residue is conserved in all the β subunit isoforms, the S383/384 residue is conserved only in the $\beta 1$ and $\beta 3$ subunits. CaMKII has also been shown to phosphorylate the $\gamma 2S$ and $\gamma 2L$ subunits at S348 and T350, and S343 in the $\gamma 2L$ subunit only (McDonald and Moss, 1994; Machu *et al.*, 1993; Table 1.1). Activation of CaMKII has also been shown to cause an increase in phosphorylation of the $GABA_A$ receptor $\alpha 1$ subunit in a synaptosomal membrane fraction (Churn *et al.*, 2002).

Further information regarding the physical and functional interaction of CaMKII with $GABA_A$ receptors can be found in Chapters 3 and 4 of this thesis, and an overview of the structure and regulation of CaMKII is provided in Section 1.8.

1.7.2.1.6 PKG

PKG is perhaps the least well characterised of the known $GABA_A$ receptor-associated serine-threonine kinases. PKG has been shown to phosphorylate the major intracellular domain of the receptor β subunits at the conserved serine residue that is also a phosphorylation site for kinases such as PKC, PKA and CaMKII, namely S410 of the $\beta 2$ subunit, and S409 of the $\beta 1$ and $\beta 3$ subunits (McDonald and Moss, 1994, 1997; Table 1.1).

1.7.2.1.7 Src

In addition to the serine/threonine kinases, $GABA_A$ receptor-associated kinases also include a member of the Src family of non-receptor tyrosine kinases. Indeed, v-src phosphorylates both purified $GABA_A$ receptors and the major intracellular domains of the receptor β1 and γ2L subunits, *in vitro* (Valenzuela *et al.*, 1995). Additional studies have shown that c-Src phosphorylates the major intracellular domain of the γ2 subunit at Y365/367, *in vitro* (Brandon *et al.*, 2001; Table 1.1). vSRC has also been shown to phosphorylate recombinant $GABA_A$ receptors expressed in HEK293 cells, and site-directed mutagenesis identified a number of phosphorylation sites, namely Y365 and Y367 of the γ2 subunit and Y370 and Y372 of the β1 subunit (Moss *et al.*, 1995; Table 1.1). Studies using phosphorylation state-specific antibodies have shown that the β2/3 subunits of the $GABA_A$ receptor are phosphorylated on tyrosine residues in cultured dorsal horn neurons (Wan *et al.*, 1997a), and that the receptor γ2 subunit is phosphorylated on Y365 and Y367 in cultured cortical neurons upon inhibition of tyrosine phosphatases (Brandon *et al.*, 2001).

An interaction between endogenous Src and the major intracellular domains of the $GABA_A$ receptor α1, β1, β2 and γ2S subunits has now been demonstrated (Brandon *et al.*, 2001; Wang *et al.*, 2003a). Although it is not yet known whether Src binds directly or indirectly to these subunits, the association of this kinase with the major intracellular domain of the γ2S subunit has been shown to involve residues 317 to 332 (Wang *et al.*, 2003a). Furthermore, although Src has been reported to bind directly to RACK-1 (Chang *et al.*, 1998), it is not yet known whether RACK-1 directs Src, as well as PKC, to $GABA_A$ receptors.

1.7.2.2 The Role of Protein Phosphatases

The relative activity of protein kinases and phosphatases is pivotal to the overall phosphorylation state of a protein. The activity of protein phosphatases at $GABA_A$ receptors is therefore a key determinant of phosphorylation-dependent modulation. The role of protein phosphatases in controlling the phosphorylation state of $GABA_A$ receptors is not as well defined as that of protein kinases. However, evidence has

emerged in recent years in support of an intimate association between $GABA_A$ receptors and various protein phosphatases.

1.7.2.2.1 PP1 and the Adaptor Protein PRIP-1

Several lines of evidence now suggest that $GABA_A$ receptors are a substrate of the serine/threonine phosphatase, PP1. Recombinant PP1α has been shown to dephosphorylate the major intracellular domain of the $GABA_A$ receptor β3 subunit that has been phosphorylated by PKA at S409, *in vitro* (Terunuma *et al.*, 2004; McDonald and Moss, 1997). Furthermore, dephosphorylation of the β3 subunit intracellular domain by hippocampal extracts has been partly attributed to endogenous PP1α activity (Terunuma *et al.*, 2004). Treatment of cultured cortical neurons with okadaic acid, at a concentration that inhibits both PP1 and PP2A, increases the basal phosphorylation of $GABA_A$ receptor β3 subunits at S408 and S409 (Jovanovic *et al.*, 2004). This indicates that one or both of these phosphatases are active at $GABA_A$ receptors under basal conditions. Furthermore, treatment of hippocampal slices with the PP1 and PP2A inhibitor, calyculin A, has been shown to cause an increase in forskolin-induced phosphorylation of the $GABA_A$ receptor β2/3 subunits, *in situ*, again implicating these phosphatases in the dephosphorylation of $GABA_A$ receptors (Terunuma *et al.*, 2004).

Coimmunoprecipitation studies have shown that PP1α forms a complex with $GABA_A$ receptors containing the β3 subunit in brain (Terunuma *et al.*, 2004). A direct interaction between PP1 and $GABA_A$ receptors has not yet been demonstrated, however it has been suggested that PP1α is targeted indirectly to these receptors in an inactive form via its interaction with the $GABA_A$ receptor-associated protein, PRIP-1 (see Section 1.6; Terunuma *et al.*, 2004; Yoshimura *et al.*, 2001; Uji *et al.*, 2002). Indeed, it has been proposed that phosphorylation of PRIP-1 by PKA induces the release of catalytically active PP1α, and dephosphorylation of the $GABA_A$ receptor β3 subunit at phospho-S408 and phospho-S409 (Terunuma *et al.*, 2004; Yoshimura *et al.*, 2001).

1.7.2.2.2 PP2A

PP2A has also been described as a $GABA_A$ receptor serine/threonine phosphatase. Purified PP2A has been shown to dephosphorylate the major intracellular domain of the $GABA_A$ receptor β3 subunit that has been phosphorylated at S409 by PKA, *in vitro* (Terunuma *et al.*, 2004; McDonald and Moss, 1997). Dephosphorylation of the β3 subunit intracellular domain by hippocampal extracts has also been partly attributed to endogenous PP2A activity, in addition to PP1α activity (Section 1.7.2.2.1), based on inhibition by okadaic acid (Terunuma *et al.*, 2004). Furthermore, as described above, PP2A has been implicated in the dephosphorylation of the $GABA_A$ receptor β3 subunit in cultured cortical neurons (Section 1.7.2.2.1; Jovanovic *et al.*, 2004).

The catalytic subunit of PP2A (PP2Ac) has been shown to form a complex with $GABA_A$ receptors containing β3 subunits in cultured cortical neurons (Jovanovic *et al.*, 2004). Affinity-purification assays have further demonstrated that neuronal PP2Ac associates with the major intracellular domain of the receptor β3 subunit, and that this interaction is enhanced when S409 is in the phosphorylated state (Jovanovic *et al.*, 2004). Treatment of cortical neurons with BDNF has also been shown to enhance binding of PP2Ac to $GABA_A$ receptors, an effect that correlates temporally with the transient BDNF-induced PKC-dependent phosphorylation of the receptor β3 subunit at S408 and S409 (Jovanovic *et al.*, 2004). The increased interaction of PP2Ac with the phosphorylated form of the β3 subunit is therefore thought to be involved in the ensuing dephosphorylation of the receptor β3 subunit (Jovanovic *et al.*, 2004).

1.7.2.2.3 PP2B

The serine/threonine phosphatase PP2B (also known as calcineurin) has also been implicated in dephosphorylating $GABA_A$ receptors. Both a recombinant and endogenous form of PP2B have been shown to dephosphorylate the major intracellular domain of the $GABA_A$ receptor γ2S subunit that has been phosphorylated at S327 by PKC, *in vitro* (Wang *et al.*, 2003a; Moss *et al.*, 1992a). PP2B has also been implicated in long-term depression (LTD)-dependent dephosphorylation of the $GABA_A$ receptor γ2 subunit at S327 in hippocampal CA1 slices (Wang *et al.*, 2003a).

However, purified PP2B does not dephosphorylate the major intracellular domain of the $GABA_A$ receptor β3 subunit that has been phosphorylated at S409 by PKA, *in vitro* (Terunuma *et al.*, 2004). Furthermore, PP2B activity is not involved in the basal dephosphorylation of $GABA_A$ receptor β3 subunits at S408/409 in cultured cortical neurons (Jovanovic *et al.*, 2004).

PP2B is believed to interact with $GABA_A$ receptors in an activity-dependent manner. In hippocampal CA1 neurons, recruitment of PP2B catalytic subunits to $GABA_A$ receptor complexes is both necessary and sufficient for inducing LTD of inhibitory synapses (Wang *et al.*, 2003a; Lu *et al.*, 2000). Coimmunoprecipitation assays from hippocampal slices have also demonstrated that PP2B and $GABA_A$ receptors do not form a native complex under basal conditions, but that the catalytic subunit of PP2B is associated with $GABA_A$ receptors containing α1, β2 and γ2 subunits after the induction of LTD (Wang *et al.*, 2003a). *In vitro* binding studies have further demonstrated that a recombinant catalytic fragment of PP2B (that exhibits calcium-independent phosphatase activity), but not the regulatory subunit of PP2B, binds directly and selectively to the major intracellular domain of the $GABA_A$ receptor γ2 subunit, but not the α1 or β2 subunits (Wang *et al.*, 2003a). The interaction with the γ2 subunit intracellular domain is dependent on amino acids 317 to 332 (Wang *et al.*, 2003a). This region includes the S327 phosphorylation site, and is involved in binding the non-receptor tyrosine kinase Src (Wang *et al.*, 2003a; Section 1.7.2.1.7). It is possible therefore, that PP2B and Src compete physically for binding to the γ2 subunit.

I previously documented that PP2B interacts with AKAP79/150 and that this anchoring protein interacts directly with the major intracellular domains of $GABA_A$ receptor β1 and β3 subunits (Brandon *et al.*, 2003; Section 1.7.2.1.2). It is perhaps surprising therefore, that affinity-purification assays from brain do not detect an (indirect) association between native PP2B and the intracellular domains of the receptor β1 and β3 subunits (Brandon *et al.*, 2003). However, it is possible that $GABA_A$ receptor β1/3 subunits bind to the same region of AKAP79/150 as PP2B and that the binding of each protein to AKAP79/150 is mutually exclusive.

1.7.3 Phosphorylation-Dependent Modulation of $GABA_A$ Receptor Function

Phosphorylation is believed to be a major means of regulating $GABA_A$ receptor function, and hence the efficacy of inhibitory neurotransmission and neuronal excitability (Moss and Smart, 1996, 2001; Sigel, 1995). Although phosphorylation-dependent functional modulation of $GABA_A$ receptors may arise from indirect phosphorylation of $GABA_A$ receptor-associated proteins (or other signalling components), evidence suggests that direct phosphorylation of receptor subunits plays a major role in this process. Indeed, direct receptor phosphorylation may alter channel activity and the cell-surface stability of $GABA_A$ receptors by altering biophysical, pharmacological and biochemical properties, such as channel open probability and open time, channel conductance, receptor desensitisation, ligand docking and receptor-protein interactions.

Phosphorylation-dependent functional modulation of $GABA_A$ receptors has been extensively investigated in heterologous cell-systems and neurons. These studies have revealed differential effects of phosphorylation that range from enhancement to inhibition of GABA-activated currents, and which are dependent upon the identity of the kinase, the site(s) of phosphorylation, the subunit composition of the receptor complex and the cell-type investigated (Moss and Smart, 1996, 2001). For example, studies of recombinant $GABA_A$ receptors expressed in heterologous cell-lines have demonstrated that activation of PKC leads to a reduction in GABA-mediated currents, and that this effect is mediated by the PKC phosphorylation sites, S409 of the β1 subunit (Krishek *et al.*, 1994; Brandon *et al.*, 2002), S410 of the β2 subunit, and S327 and S343 of the γ2 subunit (Kellenberger *et al.*, 1992; Krishek *et al.*, 1994; Table 1.1; Section 1.7.2.1.1). However, these phosphorylation sites have also been implicated in a PKC-dependent enhancement of recombinant $GABA_A$ receptor-mediated currents in L929 fibroblasts (Lin *et al.*, 1996). Similar investigations have revealed that in contrast to PKC, PKA-dependent functional modulation of $GABA_A$ receptors is not mediated by the receptor β2 subunit (McDonald *et al.*, 1998). This subunit is an *in vitro* substrate for both of these kinases, and the difference in functional modulation is believed to result from the lack of targeting of PKA to the β2 subunit in cells (Brandon *et al.*, 2003; McDonald *et al.*, 1998; Sections 1.7.2.1.1 and 1.7.2.1.2).

PKA-dependent functional modulation of $GABA_A$ receptors is therefore dependent upon the type of receptor β subunit present. Evidence suggests that the availability of either a single (S409) or dual (S408/S409) PKA phosphorylation site determines the type of modulation that occurs. Indeed, as demonstrated for PKC, PKA-induced phosphorylation of the β1 subunit at the conserved S409 residue results in a reduced GABA-mediated response (Moss *et al.*, 1992b; McDonald *et al.*, 1998). Conversely, PKA-induced phosphorylation of the β3 subunit at both the conserved S409 residue and the neighbouring S408 residue leads to potentiation of the GABA-activated response (McDonald *et al.*, 1998).

Subunit/site-specific differences in the functional modulation of $GABA_A$ receptors have also been demonstrated in studies investigating the effect of tyrosine phosphorylation on recombinant $GABA_A$ receptors expressed in HEK-293 cells. These have shown that whereas tyrosine kinase-dependent phosphorylation of the $GABA_A$ receptor β1 subunit has no apparent functional effect, tyrosine kinase-dependent phosphorylation of the γ2 subunit correlates with an enhancement of GABA-mediated currents (which is also in contrast to the negative modulation that follows PKC-dependent phosphorylation of the γ2 subunit at serine residues) (Moss *et al.*, 1995; Kellenberger *et al.*, 1992; Krishek *et al.*, 1994; see also Valenzuela *et al.*, 1995). The $GABA_A$ receptor β2 subunit also mediates tyrosine kinase-dependent positive functional modulation of recombinant $GABA_A$ receptors (Wan *et al.*, 1997a).

Cell-type-specific differences in $GABA_A$ receptor functional modulation have been observed with various protein kinases in distinct neuronal preparations. For example, PKA activity has been shown to cause a reduction of GABA-mediated currents in sympathetic ganglion neurons (Moss *et al.*, 1992b), spinal neurons (Porter *et al.*, 1990) and cerebellar granule cells (Robello *et al.*, 1993), and a decrease in the amplitude of miniature inhibitory postsynaptic currents (mIPSCs) in hippocampal pyramidal cells (Poisbeau *et al.*, 1999). Conversely, PKA has been shown to cause an enhancement of GABA-mediated currents and mIPSCs in cerebellar Purkinje cells (Kano and Konnerth, 1992), of mIPSCs in olfactory bulb granule cells (Nusser *et al.*, 1999), and of $GABA_A$ receptor-mediated currents in hippocampal dentate granule

cells (Kapur and MacDonald, 1996). Such neuron-specific differences are also exemplified with PKC. For example, activation of PKC leads to a decrease of GABA-mediated currents in sympathetic ganglion neurons (Krishek *et al.*, 1994; Brandon *et al.*, 2002) and cortical pyramidal neurons (Brandon *et al.*, 2000), but an increase in the peak amplitude of $GABA_A$ receptor-mediated mIPSCs in hippocampal dentate gyrus granule cells (Poisbeau *et al.*, 1999).

Evidence is beginning to emerge for a role of direct receptor phosphorylation in regulating the number of $GABA_A$ receptors at the cell surface. Insulin has been shown to increase the level of expression of $GABA_A$ receptors on the plasma membrane by enhancing the insertion of receptors (Wan *et al.*, 1997b). Subsequent studies have suggested that this effect involves direct Akt-dependent phosphorylation of the $GABA_A$ receptor β2 subunit at S410 (Wang *et al.*, 2003b). In addition, direct phosphorylation of the $GABA_A$ receptor β3 subunit has been suggested to regulate the internalisation of $GABA_A$ receptors from the cell surface by inhibiting the binding of the clathrin adaptor protein 2 (AP2) complex to this receptor subunit (this is discussed further in Section 5.1) (Kittler *et al.*, 2005).

1.7.4 Phosphorylation-Dependent $GABA_A$ Receptor Cross-Talk

$GABA_A$ receptors are functionally modulated following activation of various neuronal membrane receptors, and a number of distinct protein kinases and phosphatases have been implicated in such receptor cross-talk. For example, several lines of evidence suggest that PKA is involved in the modulation of $GABA_A$ receptor function by dopamine. Activation of D1 dopamine receptors in neostriatal medium spiny neurons has been shown to cause phosphorylation of $GABA_A$ receptor β1/3 subunits, and a reduction of $GABA_A$ receptor-mediated currents via a PKA/DARPP-32/PP1 signalling cascade (Flores-Hernandez *et al.*, 2000). Stimulation of these G-protein coupled receptors also leads to a PKA-mediated decrease of GABA-activated currents in olfactory bulb interneurons (Brunig *et al.*, 1999). Activation of D3 dopamine receptors in acutely dissociated neurons of the nucleus accumbens causes a reduction of GABA-mediated currents that has been attributed to inhibition of PKA (Chen *et al.*, 2006). Furthermore, stimulation of D4 dopamine receptors in globus

pallidus neurons has been shown to decrease mIPSCs via inhibition of PKA (Shin *et al.*, 2003). PKA has also been implicated in the D4 dopamine receptor-mediated inhibition of $GABA_A$ receptor-mediated currents in prefrontal cortex pyramidal neurons (Wang *et al.*, 2002), and the D5 dopamine receptor-mediated enhancement of GABA-mediated currents in cholinergic interneurons (Yan and Surmeier, 1997). In addition to PKA, dopamine-mediated modulation of $GABA_A$ receptor function has also been shown to involve PKC. Indeed, activation of D2 dopamine receptors in olfactory bulb mitral/tufted cells causes an enhancement of GABA-mediated currents via a PKC-dependent signalling cascade (Brunig *et al.*, 1999).

Serotonin-dependent modulation of $GABA_A$ receptor function also involves PKA and PKC. For example, activation of 5HT-2 receptors in prefrontal cortex pyramidal neurons produces a decrease in $GABA_A$ receptor-mediated currents that is dependent upon PKC (Feng *et al.*, 2001). Stimulation of 5-HT-4 receptors also leads to a PKA-dependent bi-directional modulation of GABAergic currents (Cai *et al.*, 2002).

PKC has been implicated in the regulation of $GABA_A$ receptor function following activation of the muscarinic acetylcholine receptor (Brandon *et al.*, 2002), in addition to the Trk B tyrosine kinase receptor (Brunig *et al.*, 2001; Jovanovic *et al.*, 2004; Boxall, 2000; Cheng and Yeh, 2003). Other kinases have also been implicated in $GABA_A$ receptor cross talk. Examples include Akt, which has been implicated in the insulin-induced positive modulation of $GABA_A$ receptor function (Wang *et al.*, 2003b; Wan *et al.*, 1997b), and CaMKII. A discussion of CaMKII-mediated regulation of $GABA_A$ receptors can be found in Section 3.1.

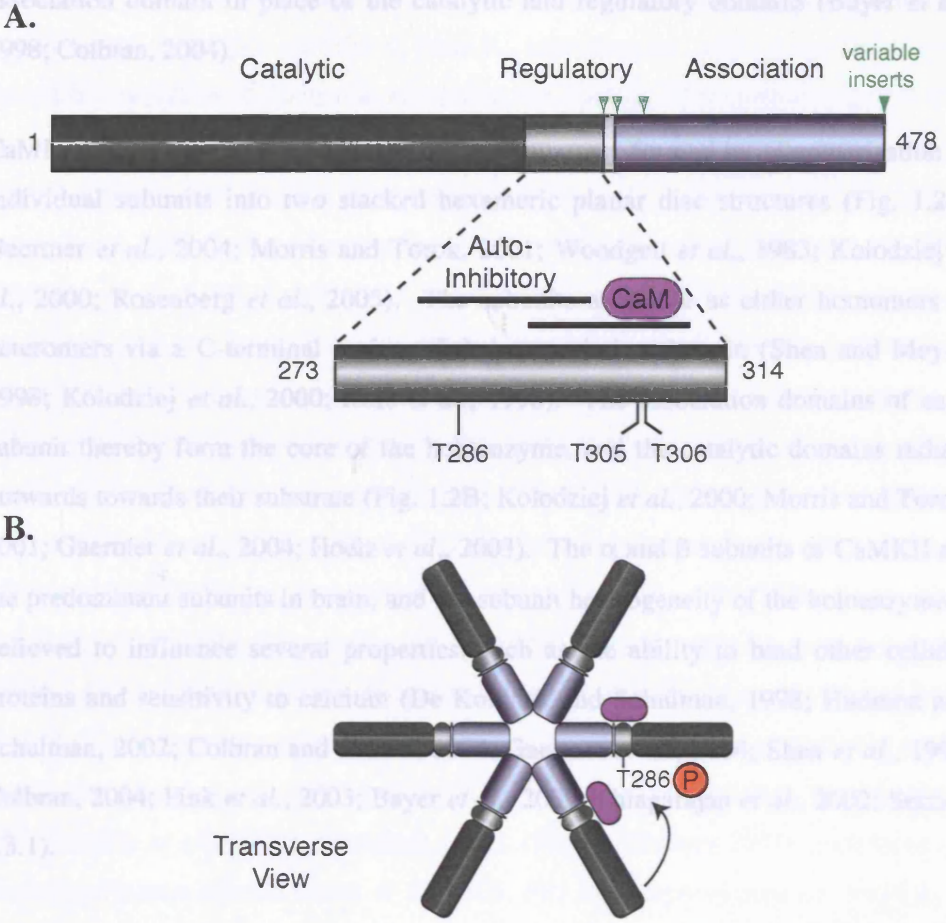
PART TWO

1.8 Structure and Regulation of CaMKII

The calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) is a ubiquitously expressed serine/threonine protein kinase, which plays a major role in transducing calcium signals in cells. This multifunctional kinase is enriched in brain, where it accounts for up to 2% of the total protein in some regions, such as the

hippocampus (Erondu and Kennedy, 1985). CaMKII is targeted to most neuronal compartments, including the postsynaptic density (PSD), dendritic shafts, the nucleus and pre-synaptic terminals (Kennedy *et al*, 1983; Hudmon and Schulman, 2002). Its activity was first described in the late 1970s (Schulman and Greengard, 1978), and subsequent work has revealed a role for this kinase in many neuronal processes, such as neurotransmission, synaptic plasticity, signal transduction, cytoskeletal organisation and gene expression (Colbran, 2004; Hudmon and Schulman, 2002). Indeed, CaMKII is believed to play a major role in the long-term regulation of excitatory glutamatergic synapses, where it has been shown to mediate the insertion of AMPA-type glutamate receptors into the plasma membrane, and to directly phosphorylate AMPA receptor subunits to potentiate channel function (Lisman *et al.*, 2002; Colbran, 2004; Hayashi *et al.*, 2000; Derkach *et al.*, 1999; Barria *et al.*, 1997). These processes are believed to be involved in animal learning and behaviour (Barria *et al.*, 1997; Lisman *et al.*, 2002; Derkach *et al.*, 1999; Colbran, 2004).

CaMKII is a large multimeric holoenzyme assembled with structurally related subunits that are encoded by four genes (α, β, γ and δ). Each subunit contains an N-terminal catalytic domain, a central regulatory domain, a C-terminal association domain and a linker region, which is located between the regulatory and association domains (Fig. 1.2A; Hudmon and Schulman, 2002). The catalytic domain is highly conserved between the subunit isoforms and the catalytic domains of other kinases, and contains the substrate- and ATP-binding sites (Colbran, 2004). The shorter regulatory domain is also highly homologous (~90% sequence identity) between the CaMKII isoforms. This region contains CaM-binding and autoinhibitory sub-domains, and several sites of autophosphorylation, including T286, T305 and T306 (Fig. 1.2A; Colbran, 2004). The association domain mediates multimerisation of the subunits into the holoenzyme complex (Shen and Meyer, 1998; Kolb *et al.*, 1998; Gaertner *et al.*, 2004; Morris and Torok, 2001; Woodgett *et al.*, 1983). Alternative mRNA splicing generates additional subunit diversity by inserting a variable cassette at up to four locations in the subunit protein (Fig. 1.2A; Hudmon and Schulman, 2002). In addition, the CaMKII isoform, αKAP, which is derived from the α gene, has been shown to contain a short hydrophobic N-terminal domain that binds the



(Adapted from Colbran, 2004)

Figure 1.2. Structure and T286-autophosphorylation of CaMKII. A. Schematic representation of the primary structure of CaMKII highlighting the different functional domains. Each of the four isoforms (α, β, γ and δ) contain an N-terminal catalytic domain (dark grey), a central regulatory domain (light grey) and a C-terminal association domain (blue). A linker region is positioned between the regulatory and association domains. Most of the diversity between the CaMKII isoforms is generated by the introduction of up to four variable inserts (green) by alternative splicing. The regulatory domain contains two overlapping sub-domains, which contain (auto)inhibitory and CaM (purple)-binding sequences, and several sites of autophosphorylation, such as T286, T305 and T306. **B.** Schematic representation of the holoenzyme structure of CaMKII highlighting the mechanism of T286-autophosphorylation. CaMKII subunits are believed to assemble via their association domains to form two hexameric planar discs that stack together to form a dodecameric structure (diagram depicts a single ring). Whilst the association domains form the core region of the holoenzyme, the kinase domains radiate outwards towards their substrate. Binding of calcium/CaM to any two adjacent subunits is required for *trans*-autophosphorylation at T286 and maximal activation.

association domain in place of the catalytic and regulatory domains (Bayer *et al.*, 1998; Colbran, 2004).

CaMKII is believed to be a dodecameric holoenzyme, formed by oligomerisation of individual subunits into two stacked hexameric planar disc structures (Fig. 1.2B; Gaertner *et al.*, 2004; Morris and Torok, 2001; Woodgett *et al.*, 1983; Kolodziej *et al.*, 2000; Rosenberg *et al.*, 2005). The subunits assemble as either homomers or heteromers via a C-terminal region of their association domain (Shen and Meyer, 1998; Kolodziej *et al.*, 2000; Kolb *et al.*, 1998). The association domains of each subunit thereby form the core of the holoenzyme, and the catalytic domains radiate outwards towards their substrate (Fig. 1.2B; Kolodziej *et al.*, 2000; Morris and Torok, 2001; Gaertner *et al.*, 2004; Hoelz *et al.*, 2003). The α and β subunits of CaMKII are the predominant subunits in brain, and the subunit heterogeneity of the holoenzyme is believed to influence several properties, such as the ability to bind other cellular proteins and sensitivity to calcium (De Koninck and Schulman, 1998; Hudmon and Schulman, 2002; Colbran and Brown, 2004; Gaertner *et al.*, 2004; Shen *et al.*, 1998; Colbran, 2004; Fink *et al.*, 2003; Bayer *et al.*, 2002; Thiagarajan *et al.*, 2002; Section 3.3.1).

The activity of CaMKII is subject to intricate regulation. Under basal conditions, CaMKII is maintained in an inactive conformational state by a number of intrasubunit interactions that occur between the autoinhibitory sub-domain (an N-terminal region of the regulatory domain) and the catalytic domain (Fig. 1.2A). These are believed to interfere with the binding of substrate molecules and ATP (Hudmon and Schulman, 2002; Colbran, 2004).

CaMKII is activated by an extracellular stimulus that leads to a rise in the intracellular calcium concentration and the level of calcium-bound CaM (calcium/CaM). Calcium/CaM binds to a C-terminal region of the regulatory domain (the CaM-binding sub-domain) that overlaps with the autoinhibitory sub-domain (Fig. 1.2; Meador *et al.*, 1993). This association is believed to disrupt the autoinhibitory interactions and therefore disinhibit the kinase (Smith *et al.*, 1992; Yang and

Schulman, 1999; Meador *et al.*, 1993; Colbran, 2004). The binding of calcium/CaM to adjacent subunits in the CaMKII holoenzyme also triggers *trans*-autophosphorylation of the kinase at a conserved residue, T286 (numbering used is of the α isoform) (Fig. 1.2; Hanson *et al.*, 1994; Bradshaw *et al.*, 2002). Autophosphorylation at T286 increases the affinity of the kinase for calcium/CaM by more than a 1000-fold, a phenomenon known as CaM trapping (Meyer *et al.*, 1992; Waxham *et al.*, 1998; Singla *et al.*, 2001). This enables calcium/CaM to remain associated with the kinase as cytosolic calcium levels return towards basal values. When calcium/CaM eventually dissociates, the kinase retains autonomous activity, an effect that is believed to be due to the continued disruption of some of the autoinhibitory interactions by the phosphorylated T286 residue, and which persists until this site is dephosphorylated by a protein phosphatase (Yang and Schulman, 1999; Colbran, 2004; Lai *et al.*, 1986; Miller and Kennedy, 1986; Miller *et al.*, 1988; Schworer *et al.*, 1986; Schworer *et al.*, 1988; Thiel *et al.*, 1988; Lou and Schulman, 1989). The stability of CaMKII's active state has contributed to this kinase being described as a 'memory molecule' (Lisman *et al.*, 2002). T286 of CaMKII can be dephosphorylated by PP1, PP2A and PP2C (Bradshaw *et al.*, 2003; Shields *et al.*, 1985; Strack *et al.*, 1997a; Fukunaga *et al.*, 1993). Whereas PP2A predominantly dephosphorylates soluble forms of CaMKII, PP1 dephosphorylates the majority of CaMKII associated with the postsynaptic density (PSD) (Shields *et al.*, 1985; Strack *et al.*, 1997a; Lisman *et al.*, 2002).

Dissociation of calcium/CaM triggers autophosphorylation of T305 or T306, and S314, which are located in the CaM-binding sub-domain, and blocks the re-binding of calcium/CaM (Hanson and Schulman, 1992; Patton *et al.*, 1990; Colbran, 1993; Colbran, 2004). Autophosphorylation of T306 is also believed to occur under basal conditions, and prevent binding of calcium/CaM. This is believed to contribute to the basal inactivity of the kinase (Colbran, 1993; Hanson and Schulman, 1992).

In addition to controlling the enzymatic activity of CaMKII, subunit autophosphorylation has also been shown to regulate the spatial localisation of the holoenzyme and interactions of the kinase with various cellular proteins (see Sections

3.1 and 3.3). *Trans*-autophosphorylation of CaMKII subunits at T286 is also believed to contribute to the function of the kinase as a decoder of calcium oscillations (De Koninck and Schulman, 1998).

PART THREE

1.9 Thesis Aims

The work presented in this thesis aims to describe molecular interactions that may be involved in regulating the functional properties and stability of $GABA_A$ receptors at the neuronal surface, and hence the efficacy of inhibitory neurotransmission and neuronal excitability. The phosphorylation state of $GABA_A$ receptors is a major determinant of receptor function, and the internalisation of $GABA_A$ receptors from the neuronal membrane to intracellular compartments plays a major role in controlling the number of receptors at the cell surface. I therefore aim to employ a broad spectrum of biochemical, and cell and molecular biological techniques to examine both physical and functional interactions between $GABA_A$ receptors and protein kinases and phosphatases, and receptor-protein interactions that are involved in the endocytosis of $GABA_A$ receptors.

I propose the following:

In Chapter 3, I aim to determine whether the protein kinase, CaMKII, binds to $GABA_A$ receptors in brain, and to examine the nature of any such interaction.

In Chapter 4, I aim to examine the reversible phosphorylation of $GABA_A$ receptors by CaMKII, *in vitro*, and to determine whether $GABA_A$ receptors are subject to CaMKII-dependent phosphorylation in neurons.

In Chapter 5, I aim to examine the interaction between the $GABA_A$ receptor and the AP2 adaptor complex, a key component of the endocytic machinery. I aim to determine whether AP2 binds to the family of $GABA_A$ receptor γ subunits, and to identify the molecular determinants of any such interactions.

CHAPTER 2

Materials and Methods

2.1 General Materials, Reagents and Equipment

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (Dorset, UK), BDH (Dorset, UK), Calbiochem (Nottingham, UK) or Tocris (Bristol, UK). Redivue™ [γ-^{32}P]-ATP (~3000 Ci/mmol; 10 mCi/ml), [^{33}P]-orthophosphate (>2500 Ci/mmol; 10 mCi/ml), Redivue™ PRO-MIX™ L-[^{35}S] *in vitro* Cell Labelling Mix (>1000 Ci/mmol; 14.3 mCi/ml) and [^{125}I]-labelled anti-rabbit whole antibody from donkey (12.7 mCi/mg; 100 µCi/ml) were obtained from Amersham Biosciences (Buckinghamshire, UK). All tissue culture reagents were purchased from Invitrogen/Gibco (Paisley, UK). All enzymes for the manipulation of DNA were bought from Promega (Madison, WI, USA) or New England Biolabs (Beverly, MA, USA). All oligonucleotides were purchased from MWG Biotech (Milton Keynes, UK). All centrifugations, unless otherwise stated, were carried out in an Eppendorf 5415-D bench-top centrifuge or a 5415-R refrigerated centrifuge at 4 °C. All absorbance readings were measured using a Bio-Rad SmartSpec 3000 spectrophotometer.

2.2 Bacteria

Standard bacterial manipulations were performed as described in Sambrook *et al.*, 1989.

2.2.1 *Escherichia coli* Strains

Table 2.1 lists the genotypes and sources of the *E. coli* strains used in this study.

2.2.2 Media, Growth and Storage

Bacteria cultures were grown at 37 °C in sterile Luria-Bertani (LB) medium (tryptone (10 g/l), yeast extract (5 g/l), NaCl (10 g/l) and pH to 7.5 with NaOH) with shaking at 225-250 rpm, or on LB-agar plates (LB with agar (12 g/l)). Plasmid selection was maintained by adding chloramphenicol (Chloramp) to a final concentration of 25 µg/ml (for BL21 (DE3) pLysS) and/or ampicillin (Amp) (for BL21 (DE3) pLysS, DH5α, DH10B, MC1061 and XL1-Blue) to a final concentration of 50 µg/ml. For short-term storage, bacterial cultures were kept on LB-agar plates at 4 °C, and for long-term storage they were kept in LB medium with 20% (v/v) glycerol, at -80 °C.

Table 2.1. Strains of *E. coli* used in this study.

Strain	Genotype	Source
BL21 (DE3) pLysS	F⁻ *ompT* *hsd*S_B (r_B^- m_B^-) *dcm gal* λ(DE3) pLysS Cm^r	Promega
DH5α	F⁻ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1* *endA1* *hsdR17*(r_k^- m_k^+) *phoA* *supE44* *thi-1* *gyrA96* *relA1* λ⁻	Invitrogen
DH10B	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74* *recA1* *endA1* *ara*D139 Δ(*ara leu*)7697 *galU* *galK* λ⁻ *rpsL* *nupG*	Invitrogen
MC1061	F⁻ *hsdR* (r_k^- m_k^+) *ara*D139 Δ(*ara*ABC-*leu*)7679 *galU* *galK* Δ*lacX74* *rpsL* *thi* *mcrB*	Invitrogen
XL1-Blue	*recA1* *endA1* *gyrA96* *thi-1* *hsdR17* *supE44* *relA1* *lac* [F'*pro*AB *lac*I^qZΔM15 Tn10 (Tet^r)]	Stratagene

2.2.3 Transformation

2.2.3.1 Heat-Shock

BL21 (DE3) pLysS, DH5α, MC1061 or XL1-Blue competent cells were thawed on ice (Table 2.1). A 100-μl aliquot of cells was mixed with between 1 and 50 ng of DNA in a chilled Falcon polypropylene tube and incubated on ice for 30 min. The cells were heat-shocked for 45 s in a 42 °C water bath and then immediately incubated for 2 min on ice. 0.9 ml of warm (37 °C) LB media was then added and the tube was incubated for 1 h at 37 °C. Dilutions of the transformation mixture were plated on LB-agar plates containing the appropriate antibiotic. Plates were incubated overnight at 37 °C.

2.2.3.2 Production of Electro-Competent Cells

10 ml of LB medium was inoculated with a single colony of DH10B cells and incubated overnight at 37 °C. The overnight culture was transferred to 1 l of LB medium (1/100 dilution) and grown at 37 °C until the absorbance at 600 nm reached 0.6 (A_{600} = 0.6). The bacteria were harvested in chilled tubes by centrifugation at 3,000 rpm for 30 min at 4 °C. The cells were washed twice in 500 ml of chilled water, and once in 20 ml of chilled 10% (v/v) glycerol. The final pellet was re-suspended in 2.5 ml of chilled 10% (v/v) glycerol. 100-μl aliquots were 'snap-frozen' in liquid nitrogen, and stored at -80 °C until required.

2.2.3.3 Electroporation

Electro-competent DH10B cells were thawed on ice (Table 2.1). A 50-µl aliquot was mixed with 0.1 ng of vector DNA (or 1 µl of pUC18 control DNA (0.1 ng/µl)) (Table 2.2) and transferred to a 0.2-cm electroporation cuvette (Equibio; Middlesex, UK). The bacteria were electroporated using a Bio-Rad Gene Pulser II (single pulse; 2.5 kV, 200 Ω and 25 µF), and were immediately re-suspended in 950 µl of warm (37 °C) LB medium and incubated for 1 h at 37 °C. Dilutions of the transformation mixture (or 2.5 µl of the control mixture) were plated onto LB-agar plates containing the appropriate antibiotic (LB-Amp-agar plates were used for the control) and incubated overnight at 37 °C. The number of colonies obtained from the test transformation was compared to that from the control transformation, which was expected to produce 250 colonies (for the maximal transformation efficiency of $\geq 1 \times 10^9$ colony forming units per µg of pUC18 DNA).

2.2.4 Production and Purification of Glutathione S-Transferase-Fusion Proteins

10 ml of sterile LB-Amp/Chloramp medium was inoculated with a single colony of BL21 (DE3) pLysS bacteria transformed with the appropriate pGEX vector (Tables 2.1 and 2.2; Section 2.2.3), and incubated at 37 °C overnight.

The following day, the culture was added to 1 l of LB-Amp/Chloramp medium and grown at 37 °C, until A_{600} = 0.4-0.6 (approx 2.5 h). Isopropylthio-b-D-galactoside (IPTG) was then added to a final concentration of 0.5 mM, to induce protein expression, for a further 3 h at room temp. The cells were harvested by centrifugation at 4,000 rpm for 15 min at 4 °C, re-suspended in 10 ml of chilled Buffer A (50 mM Tris pH 8, 25% (w/v) sucrose and 10 mM EDTA) and re-centrifuged. The final pellet was drained and stored overnight at -20 °C.

The next day, the pellet was re-suspended in 10 ml of chilled Buffer B (10 mM Tris pH 7.4, 1 mM EDTA, 1% (v/v) triton-X-100, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.4% (v/v) aprotinin, and 1 µg/ml each of antipain, leupeptin and pepstatin) and the suspension was sonicated, on ice, for 30 s, five times, to lyse cells and shear the DNA. Following addition of 25 ml of chilled

Buffer C (20 mM HEPES, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 0.4% (v/v) aprotinin and 1 μg/ml each of antipain, leupeptin and pepstatin), the lysate was centrifuged in a Beckman L-70 ultracentrifuge (45 Ti rotor), at 35,000 rpm for 30 min at 4 °C. The supernatant was then mixed with 2 ml of glutathione-agarose beads (hydrated in water, and washed and retained as a 50% (v/v) slurry in Buffer C) and rotated for 1 to 2 h at 4 °C. The beads were then centrifuged at 2,000 rpm for 2 min at 4 °C, and washed 3 times in 5 ml of chilled Buffer C. In the final wash step, the beads were re-suspended in 1 ml of chilled Buffer C, and transferred to a microfuge tube.

To elute the glutathione S-transferase (GST)-fusion proteins from the beads, samples were centrifuged at 6,500 rpm for 2 min at 4 °C, and the supernatant was discarded. After addition of 1 ml of chilled Buffer D (Buffer C with 70 mM reduced glutathione, and pH to 7.5 with NaOH), the solution was re-centrifuged and the supernatant was retained. The beads were then mixed with a further 1 ml of chilled Buffer D for 15 min at 4 °C. After centrifuging again, the supernatant was pooled with that previously obtained. The eluted protein was transferred to a 0.5-3-ml dialysis cassette (Pierce; Rockford, IL, USA), and dialysed against 2 l of phosphate buffered saline (PBS) (NaCl (8 g/l), KCl (0.2 g/l), disodium hydrogen orthophosphate (1.15 g/l), potassium dihydrogen orthophosphate (0.2 g/l), and pH to 7.2 with HCl) overnight at 4 °C to remove impurities and to enable an accurate determination of concentration. The following morning, the buffer was replaced, and dialysis was continued for a further 2 h. The proteins were transferred to a microfuge tube and glycerol was added to a final concentration of 25% (v/v).

The protein concentration of the GST-fusion protein preparation was measured using the Bradford protein assay (Section 2.5.4.2). The GST-fusion proteins, and any protein contaminants, were visualised and assessed for degradation following Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and staining with Brilliant Blue R (Sections 2.5.5 and 2.5.6). 100-μl aliquots of the GST-fusion proteins were 'snap-frozen' in liquid nitrogen and stored at -80 °C until required.

2.2.5 Purification of Plasmid DNA from *E. coli*

2.2.5.1 Mini-Preparation of Plasmid DNA

A Strataprep® Plasmid Miniprep Kit (Stratagene; CA, USA) was used to extract and purify approx 20 μg of plasmid DNA from a bacterial culture for use in restriction digestions, ligations and automated sequencing reactions. Briefly, 5 ml of LB medium containing the appropriate antibiotic was inoculated with a single bacterial colony, and incubated overnight at 37 °C. To harvest the bacteria, 1.5 ml of the cell culture was transferred into a microfuge tube and centrifuged at 13,000 rpm for 1 min. Plasmid DNA was recovered in accordance with the manufacturer's guidelines. The DNA yield and purity was determined by optical density measurement using a Bio-Rad SmartSpec 3000 spectrophotometer (Section 2.3.5). Where appropriate, plasmid DNA was further purified using ethanol and phenol/chloroform extractions (Sections 2.3.4.1 and 2.3.4.2). Aliquots of plasmid DNA were stored at 4 °C, in the short-term, and at -20 °C, in the long-term.

2.2.5.2 Maxi-Preparation of Plasmid DNA

A HiSpeed™ Plasmid Maxi Kit (QIAGEN; West Sussex, UK) was used to isolate and purify approx 750 μg of plasmid DNA from a bacterial culture for use in transfections, enzymatic modifications and automated sequencing reactions. Briefly, 2 ml of LB medium containing the appropriate antibiotic was inoculated with a single bacterial colony and incubated for approx 8 h at 37 °C. 0.3 ml of the starter culture was diluted into 150 ml of selective LB medium and grown for 12-16 h at 37 °C, with vigorous shaking. The bacterial cells were harvested by centrifugation in a Beckman J6-MC centrifuge (rotor JS 4.2) at 4,000 rpm for 15 min at 4 °C. Plasmid DNA was then recovered in accordance with the manufacturer's guidelines.

The DNA yield and purity was determined by optical density measurement using a Bio-Rad SmartSpec 3000 spectrophotometer (Section 2.3.5). Where appropriate, the plasmid DNA was further purified using ethanol and phenol/chloroform extractions (Sections 2.3.4.1 and 2.3.4.2). Aliquots of plasmid DNA were stored at 4 °C, in the short-term, and at -20 °C, in the long-term.

2.2.5.3 Large-Scale Preparation of Plasmid DNA

Caesium chloride/ethidium bromide fractionation was used to purify a large quantity (typically mg) of high-quality plasmid DNA from a cleared bacterial lysate for use in transfections, enzymatic modifications and automated sequencing reactions. 10 ml of LB medium containing the appropriate antibiotic was inoculated with a single bacterial colony and incubated for 12-16 h at 37 °C. The starter culture was then transferred to 1 l of selective LB medium and grown overnight at 37 °C. The bacteria were harvested by centrifugation at 4,000 rpm in a J6-MC centrifuge (rotor JS 4.2) for 15 min at 4 °C, and re-suspended in 40 ml of solution I (50 mM glucose, 25 mM Tris, pH 8, and 10 mM EDTA). 20 ml of solution II (1% (w/v) SDS and 200 mM NaOH) was then added, and the lysate was mixed gently and left on ice for 5 min. 15 ml of solution III (5 M potassium acetate) was then added, and the suspension was incubated on ice for a further 5 min. A cleared lysate was obtained following centrifugation at 3,000 rpm in a J6-MC centrifuge for 20 min at room temp. DNA was precipitated upon addition of an equal volume of isopropanol, and pelleted by centrifugation at 3,000 rpm for 10 min at room temp.

To further purify the plasmid DNA, the pellet was re-suspended in 10 ml of 10X Tris-EDTA (TE) (10 mM Tris-Cl, pH 7.6, and 1 mM EDTA, pH 8) and mixed with 1 ml of 5 M ammonium acetate and 2 volumes of approx 100% (v/v) ethanol. Following centrifugation at 3,000 rpm for 10 min at room temp, the pellet was re-suspended in 6 ml of 10X TE, and exactly 1.1 g of caesium chloride was added per ml of supernatant. 100 µl of ethidium bromide (10 mg/ml) was added, and the mixture was transferred to two Beckman Quick-Seal™ tubes, and centrifuged in a Beckman optima™ ultracentrifuge (TLN 100 rotor) at 100,000 rpm overnight at room temp. The lower band of covalently closed, circular, plasmid DNA was extracted from each sample using a syringe and hypodermic needle, and pooled. To remove the ethidium bromide, 1 volume of water-saturated butanol was added, mixed well, and left to stand to allow the two phases to separate. The upper phase, containing the ethidium bromide, was discarded and the process was repeated until all the ethidium bromide was removed.

The plasmid DNA was then subjected to two ethanol precipitations (Section 2.3.4.1), followed by a phenol/chloroform extraction (Section 2.3.4.2) and a further ethanol precipitation. The DNA pellet was re-suspended in 1 ml of sterile water, and the yield and purity of the DNA was determined (Section 2.3.5). Aliquots of plasmid DNA were stored at 4 °C, in the short-term, or at -20 °C, in the long-term.

2.3 Molecular Biology

2.3.1 DNA Manipulations

All DNA manipulations were performed as described in Sambrook *et al.*, 1989.

2.3.2 Vectors

The DNA vectors that were used in this study are described in Table 2.2.

Table 2.2. Vectors used in this study.

Vector	Description	Source
pUC18	Small, high copy number *E. coli* cloning vector	Stratagene
pcDNA3	Mammalian expression vector containing a cytomegalovirus promoter and designed for high-level expression of cloned genes	Invitrogen
pcDNA3-CaMKII-WT	CaMKIIα subunit cloned into pcDNA3	H. Schulman
pcDNA3-CaMKII-TD	CaMKIIα-T286D subunit cloned into pcDNA3	H. Schulman
pcDNA3-CaMKII-TA	CaMKIIα-T286A subunit cloned into pcDNA3	H. Schulman
pRK5	Mammalian expression vector containing a cytomegalovirus promoter and designed for high-level expression of cloned genes	BD Pharmingen
pRK5-β3-S383A	$GABA_A$ receptor β3-S383A subunit cloned into pRK5	T. Smart
pRK5-β3-S383A/ S409A	$GABA_A$ receptor β3-S383A/S409A subunit cloned into pRK5	K. McAinsh
pGEX-2T	GST gene fusion vector containing a tac promoter for chemically inducible, high-level expression in any *E. coli* host	Amersham Biosciences

Vector	Description	Source
pGEX-4T1/ pGEX-4T2/ pGEX-4T3	GST gene fusion vectors derived from pGEX-2T with an expanded multiple cloning site	Amersham Biosciences
pGEX-4T-α1	$GABA_A$ receptor α1 subunit ICD cloned into pGEX-4T3	S.J. Moss
pGEX-4T-β1	$GABA_A$ receptor β1 subunit ICD cloned into pGEX-4T3	S.J. Moss
pGEX-4T3-β2	$GABA_A$ receptor β2 subunit ICD cloned into pGEX-4T3	S.J. Moss
pGEX-4T3-β3	$GABA_A$ receptor β3 subunit ICD (304-426) cloned into pGEX-4T3	S.J. Moss
pGEX-4T2-β3-N	$GABA_A$ receptor β3 subunit ICD (residues 304-365) cloned into pGEX-4T2	S.J. Moss
pGEX-4T2-β3-C	$GABA_A$ receptor β3 subunit ICD (residues 366-426) cloned into pGEX-4T2	S.J. Moss
pGEX-4T2-β3-Q1	$GABA_A$ receptor β3 subunit ICD (residues 304-333) cloned into pGEX-4T2	S.J. Moss
pGEX-4T2-β3-Q2	$GABA_A$ receptor β3 subunit ICD (residues 334-365) cloned into pGEX-4T2	S.J. Moss
pGEX-4T2-β3-Q3	$GABA_A$ receptor β3 subunit ICD (residues 366-395) cloned into pGEX-4T2	S.J. Moss
pGEX-4T2-β3-Q4	$GABA_A$ receptor β3 subunit ICD (residues 396-426) cloned into pGEX-4T2	S.J. Moss
pGEX-4T3-β3-T1	$GABA_A$ receptor β3 subunit ICD (residues 313-420) cloned into pGEX-4T3	K. McAinsh
pGEX-4T3-β3-T2	$GABA_A$ receptor β3 subunit ICD (residues 323-420) cloned into pGEX-4T3	K. McAinsh
pGEX-4T3-β3-T3	$GABA_A$ receptor β3 subunit ICD (residues 332-420) cloned into pGEX-4T3	K. McAinsh
pGEX-4T-β3 (345-408)	$GABA_A$ receptor β3 subunit ICD (residues 345-408) cloned into pGEX-4T	S.J. Moss
pGEX-4T3-β3-S383A	$GABA_A$ receptor β3-S383A (residues 304-420) subunit ICD cloned into pGEX-4T3	K. McAinsh
pGEX-4T-β3-S408A	$GABA_A$ receptor β3-S408A subunit ICD cloned into pGEX-4T3	S.J. Moss
pGEX-4T-β3-S409A	$GABA_A$ receptor β3-S409A subunit ICD cloned into pGEX-4T3	S.J. Moss
pGEX-4T3-β3-S408A/S409A	$GABA_A$ receptor β3-S408A/S409A subunit ICD cloned into pGEX-4T3	S.J. Moss
pGEX-4T3-β3-S383A/S409A	$GABA_A$ receptor β3-S383A/S409A (residues 304-420) subunit ICD cloned into pGEX-4T3	K. McAinsh
pGEX-4T3-β3-S383A/S408A/S409A	$GABA_A$ receptor β3-S383A/S408A/S409A subunit ICD cloned into pGEX-4T3	S.J. Moss
pGEX-4T1-γ1	$GABA_A$ receptor γ1 subunit ICD cloned into pGEX-4T1	J. Anderson

Vector	Description	Source
pGEX-2T-γ2S	$GABA_A$ receptor γ2S subunit ICD (full-length, residues 318-404) cloned into pGEX-2T	S.J. Moss
pGEX-2T-γ2S-N	The N-half of the $GABA_A$ receptor γ2S subunit ICD (318-362) cloned into pGEX-2T	S.J. Moss
pGEX-2T-γ2S-C	The C-half of the $GABA_A$ receptor γ2S subunit ICD (363-404) cloned into pGEX-2T	S.J. Moss
pGEX-4T3-γ2S (327-404)	$GABA_A$ receptor γ2S subunit ICD (327-404) cloned into pGEX-4T3	K. McAinsh
pGEX-4T3-γ2S (338-404)	$GABA_A$ receptor γ2S subunit ICD (338-404) cloned into pGEX-4T3	K. McAinsh
pGEX-4T3-γ2S (347-404)	$GABA_A$ receptor γ2S subunit ICD (347-404) cloned into pGEX-4T3	K. McAinsh
pGEX-2T-γ2L	$GABA_A$ receptor γ2L subunit ICD cloned into pGEX-2T	S.J. Moss
pGEX-4T1-γ3	$GABA_A$ receptor γ3 subunit ICD cloned into pGEX-4T1	J. Anderson
pGEX-4T2-δ	$GABA_A$ receptor δ subunit ICD cloned into pGEX-4T2	S.J. Moss
pGEX-4T-EGFR	EGF receptor intracellular domain cloned into pGEX-4T	V. Haucke
pcDNA3-α adaptin	~100 kDa α adaptin of the AP2 complex	V. Haucke
pcDNA3-β2 adaptin	~100 kDa β2 adaptin of the AP2 complex	V. Haucke
pcDNA3-μ2 adaptin	50 kDa μ2 adaptin (residues 1-435) of the AP2 complex	V. Haucke
pcDNA3-μ2 (158-435),	μ2-adaptin, residues 158-435	V. Haucke
pcDNA3-μ2 (158-407)	μ2-adaptin, residues 158-407	V. Haucke
pcDNA3-μ2 (283-394)	μ2-adaptin, residues 283-394	V. Haucke
pcDNA3-σ2 adaptin	17 kDa σ2 adaptin of the AP2 complex	V. Haucke

ICD = major intracellular domain

2.3.3 Oligonucleotides

The oligonucleotides that were used in this study are listed in Table 2.3.

2.3.4 Purification of Plasmid DNA

2.3.4.1 Ethanol Precipitation

DNA was precipitated from an aqueous solution by first adding 0.1 volume of 3 M

Table 2.3. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′ to 3′)
β3-S409A-Mutagenesis1	CACCTACGGAGGAGGTCTGCCCAGCTCAAAATC
β3-S409A-Mutagenesis2	GATTTTGAGCTGGGCAGACCTCCTCCGTAGGTG
β3-ICD(304-)-Forward	CGCCGCGGATCCAACTACATTTTCTTTGGAAG
β3-ICD(-420)-Reverse	CATCATCTCGAGAGTCATCGGTTAGATCAGGG
β3-ICD-T1(313-)-Forward	CGCCGCGGATCCCAAAGGCAGAAGAAGCTTGC
β3-ICD-T2(323-)-Forward	CGCCGCGGATCCGCCAAGGCCAAGAATGATCG
β3-ICD-T3(332-)-Forward	CGCCGCGGATCCGAAATAAACCGGGTGGATGC
γ2S-ICD(327-)-Forward	CGCGGATCCAAGGATAAAGACAAAAAG
γ2S-ICD(338-)-Forward	CGCGGATCCGCCCCTACCATTGATATTC
γ2S-ICD(347-)-Forward	CGCGGATCCGCAACCATTCAAATGAAC
γ2S-ICD(-404)-Reverse	CGGAATTCGGAGTCCATTTTGGCAATG

ICD=major intracellular domain

NaOAc, pH 5.2, and then two volumes of approx 100% (v/v) ethanol. The solution was incubated for 10 min at -20 °C and centrifuged at 13,000 rpm for 15 min. The pellet was washed in 500 µl of 70% (v/v) ethanol and air-dried at room temp.

2.3.4.2 Phenol/Chloroform Extraction

DNA was purified from protein by adding 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1; ICN Biomedicals; Aurora, OH, USA) to the DNA solution and vortexing. Following centrifugation at 13,000 rpm for 5 min at room temp, the aqueous phase was transferred to a new microfuge tube. The process was then repeated with an equal volume of chloroform/isoamyl alcohol (24:1), and followed by an ethanol precipitation (Section 2.3.4.1).

2.3.5 Measurement of DNA Concentration and Purity

The absorbance of a diluted DNA sample was measured at 260 nm and 280 nm. The

formula for calculating the concentration of DNA, where A_{260} is the absorbance at 260 nm, and f is the dilution factor, is given by

$$[\text{DNA } \mu g / ml] = 50 A_{260} f \qquad (2.1)$$

The purity of the DNA solution was measured by calculating the ratio A_{260}/A_{280}. Samples with a value greater than 1.8 were used in further DNA manipulations.

2.3.6 Polymerase Chain Reaction

Each polymerase chain reaction (PCR) reaction was assembled in a 0.2-ml thin-walled tube on ice in a total volume of 50 µl, as follows: 5 µl of *Pfu* DNA Polymerase 10X buffer with $MgSO_4$, 250 µM of each deoxynucleotide triphosphate (dNTP; dATP, dCTP, dGTP, dTTP), 50 pmol of the upstream primer, 50 pmol of the downstream primer, 40 ng of template DNA, and 2 u of *Pfu* DNA polymerase. The amplification reaction was performed in a PTC-200 Peltier Thermal Cycler with a heated lid, in accordance with the following programme: initial denaturation at 94 °C for 1 min (1 cycle); further denaturation at 94 °C for 30 s, annealing at 45-65 °C for 30 s and extension at 72 °C for 1 min (30 cycles); a final extension at 72 °C for 5 min (1 cycle). The annealing temp depended upon primer melting temp. The extension time depended upon the extension rate of *Pfu* DNA polymerase (approx 2 min per kb of DNA).

DNA fragments were purified from the PCR reaction using a QIAquick® PCR purification kit (QIAGEN), in accordance with the manufacturer's guidelines. PCR products were stored at 4 °C following analysis by agarose gel electrophoresis (Section 2.3.7).

2.3.7 Agarose Gel Electrophoresis

DNA samples were mixed with 0.2 volumes of 6X Loading Buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 30% (v/v) glycerol), and loaded into gels comprising 1% (w/v) agarose in 1X TAE (Tris-Acetate EDTA; 40 mM Tris-acetate and 1 mM EDTA, pH 8) and ethidium bromide at a concentration of

0.15 μg/ml. A 1-kb DNA ladder (0.1 μg/μl in 1X Loading Buffer) was loaded as a standard. DNA was resolved at 120 V in 1X TAE, and visualised using a UV transilluminator.

2.3.8 Site-Directed Mutagenesis

In vitro site-directed mutagenesis was performed using the Stratagene QuikChange® mutagenesis kit, in accordance with the manufacturer's guidelines. The oligonucleotide primers used were designed individually according to the desired mutation and are listed in Table 2.3.

2.3.9 Restriction Digests

A double restriction enzyme digest was set up in a 0.2-ml thin-walled tube on ice, in a total volume of 20 μl, as follows: 2 μl of the appropriate 10X buffer (provides conditions amenable to both restriction enzymes to be used), 2 μg of acetylated bovine serum albumin (BSA), 1 μg of DNA (or 5 μl of PCR product DNA), and 5 u each of restriction endonucleases 1 and 2 (5-fold excess of enzyme over DNA). The reaction was incubated at 37 °C for 1 to 2 h. The digested samples were resolved using agarose gel electrophoresis (Section 2.3.7). A QIAquick gel extraction kit (QIAGEN) was used in accordance with the manufacturer's guidelines to extract and purify the DNA from the agarose gel for further DNA manipulations. If desired, the digested DNA was stored at -20 °C until required.

2.3.10 Ligations

Ligation reactions were set up in a 0.2-ml thin-walled tube on ice in a total volume of 20 μl. The concentration of both vector and insert DNA was approximated on the basis of their band intensity on an agarose gel. A vector/insert molar ratio of 1:3 or 1:5 was typically used. Ligation reactions were assembled as follows: 2 μl of 10X T4 DNA Ligase Buffer, digested vector DNA (approx 100 ng), digested insert DNA (amount as appropriate) (or 3 μl of water as a control), 1 μl of 10 mM ATP, pH 7.5, and between 0.1 and 1 u of T4 DNA ligase. The reaction was incubated overnight at 4 °C. 1 μl of the ligation reaction was then used to transform XL1-Blue cells (Section 2.2.3; Table 2.1). Mini-preps (Section 2.2.5.1) were performed if a two-fold

enhancement in the number of colonies was detected with respect to the control reaction. Restriction digests (Section 2.3.9) were performed at the cloning sites using the mini-prep DNA to verify the presence of the insert before DNA sequence analysis (Section 2.3.11).

2.3.11 DNA Sequence Analysis

All constructs were checked by DNA sequence analysis at the Automated DNA Sequencing Service at Cytomyx (Cambridge, UK) or the MRC Gene Service (Cambridge, UK). Sequencing data was analysed using DNA Strider 1.2 (by Christian Marck; Gif-sur-Yvette, France) and TraceViewer (CodonCode; Dedham, MA, USA) software.

2.4 Cell Biology

2.4.1 Manipulations using COS-7 Cell Lines

2.4.1.1 COS-7 Cell Lines

The African green monkey (*Cercopithecus aethiops*) kidney (COS-7) cell line (ATCC; Manassas, VA, USA) was used as a transfection host. It is derived from the CV-1 cell line by transformation with an origin-defective mutant of SV40 (Gluzman, 1981). COS-7 cells have a fibroblast-like morphology, are adherent and grow as monolayers.

2.4.1.2 Propagation of COS-7 Cell Lines

A vial of COS-7 cells was removed from the liquid nitrogen store and placed in a 37 °C water bath to defrost. An aliquot of cells was transferred to Dulbecco's Modified Eagle Medium (DMEM) with high glucose, which was supplemented with 2 mM L-glutamine, penicillin (100 u/ml)/ streptomycin (100 μg/ml) and 10% (v/v) foetal bovine serum (FBS), and was incubated at 37 °C, 5% CO_2 and 100% humidity. Cells were grown as monolayers in 100-mm dishes (Nunc; Rochester, NY, USA), and passaged at approx 80% confluence.

COS-7 cells were subcultured two to three times per week: the medium was aspirated, and the cells were rinsed twice in 2 ml of trypsin/EDTA (NaCl (8 g/l), KCl (0.2 g/l), disodium hydrogen orthophosphate (1.15 g/l), potassium dihydrogen orthophosphate (0.2 g/l), trypsin (2.5 g/l), EDTA (0.2 g/l) and phenol red (0.0015 g/l)). A further 2 ml of trypsin was added and cells were incubated for 5 min at 37 °C. COS-7 cells were re-suspended in 8 ml of supplemented DMEM, and an appropriate aliquot of the cell suspension was transferred to fresh supplemented DMEM (the cell suspension was usually diluted 1/10). If desired, aliquots of the cell suspension were 'snap-frozen' in supplemented DMEM and kept in a liquid nitrogen store until required.

2.4.1.3 Transient Transfection of COS-7 Cell Lines

Two 100-mm dishes of COS-7 cells were used per transfection, when the cells were approx 80 % confluent. Cells were trypsinised (Section 2.4.1.2) and re-suspended in 5 ml of supplemented DMEM. Following centrifugation at 2000 rpm for 2 min at room temp, cells were re-suspended in 10 ml of optimem, centrifuged again and re-suspended in another 0.5 ml of optimem. Cells were transferred to a sterile 0.4-cm electroporation cuvette (Equibio) and mixed with 10 μg of the plasmid DNA of interest (Table 2.2). Cells were then electroporated using a Bio-Rad Gene Pulser II (2 pulses; 400 V, ∞ Ω and 125 μF; time constant between 5 and 6), and transferred to 6 ml of supplemented DMEM in a 60-mm dish (Nunc). Transfected cells were maintained for 24 h before use.

2.4.2 Primary Neuronal Culture

2.4.2.1 Rat Species

Sprague-Dawley female rats (UCL breeding colony) were obtained from the UCL animal facility. Dissections were performed in compliance with UK Home Office regulations and UCL guidelines.

2.4.2.2 Preparation of Dishes

100-mm dishes (Nunc) were treated with poly-D-lysine (0.1 mg/ml) for a minimum of 45 min at 37 °C, washed twice in sterile water, and incubated for at least 1 h in

DMEM supplemented with 10% (v/v) FBS and penicillin (100 u/ml)/ streptomycin (100 μg/ml). The medium was aspirated immediately before plating the neurons.

2.4.2.3 Preparation of Primary Rat Cortical Neurons

Dissociated cortical neurons were prepared following a protocol modified from Banker and Goslin, 1998, and by using a Nikon C-DS dissecting microscope and appropriate surgical instruments (FST; Heidelberg, Germany). Briefly, brains were removed from embryonic day 17 (E17) rat embryos and placed in chilled, sterile PBS. Following removal of the meninges, cortices were isolated from the rest of the brain, and placed in 1 ml of supplemented neurobasal medium (neurobasal medium with 2% (v/v) (1X) B27 supplement, 2 mM L-glutamine, penicillin (100 u/ml)/ streptomycin (100 μg/ml) and 0.6% (w/v) D-+-glucose). Neurons were dissociated by trituration with a flame-polished glass Pasteur pipette, and the number of viable cells were estimated using 0.4% (w/v) Trypan Blue Solution and an Improved Neubauer haemocytometer. Neurons were plated at high density (approx 12 million cells per 100-mm dish) in an appropriate volume of supplemented neurobasal medium, and were maintained at 37 °C, 5% CO_2 and 100% humidity until required (6-8 DIV).

2.4.3 Production of Phosphorylation State-Specific Antibodies

2.4.3.1 Production of Synthetic Peptides for Immunisation of Rabbits and Characterisation of Phosphorylation State-Specific Antisera

All peptides used in this study (Table 2.4) were synthesised and HPLC purified at The Rockefeller University Protein/DNA Technology Centre, NY, USA, (www.pdtc.rockefeller.edu). Each peptide was designed in accordance with the Centre's guidelines, and comprised a sequence of the $GABA_A$ receptor β3 subunit that surrounded either S383 or S408/S409 (Table 2.4). The peptides were modified, where appropriate, with a chemically phosphorylated serine residue, and an N-terminal cysteine residue for conjugation to the *Limulus* hemocyanin carrier-protein, via the cross-linker, sulfo-MBS (*m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester) (Table 2.4; Section 2.4.3.2). The β3-S383 and β3-S383-P peptides were designed and synthesised in this study. Steve Moss provided the other peptides.

2.4.3.2 Conjugation of Peptide/Carrier Proteins with Sulfo-MBS

The cysteine-containing β3-S383-P and β3-S409-P phospho-peptides were conjugated to the carrier protein, *Limulus* hemocyanin, using the hetero-bifunctional cross-linker sulfo-MBS. The carrier protein was dissolved to 2 mg/ml in Coupling Buffer (75 mM Na phosphate, pH 7.2, and 250 mM NaCl) and the solution was passed through an Acrodisc 0.45-μm-syringe filter (Pall; MI, USA). The cross-linker, sulfo-MBS, was dissolved to 2.5 mg/ml in Coupling Buffer immediately prior to use. 125 μl of the sulfo-MBS solution was added to 1.25 ml of the carrier protein solution and incubated for 60 min at room temp to first couple the NHS moiety of the cross-linker to free amino groups (NH_2-terminal and/or ε-amino groups of lysine residues) in the carrier protein. During the carrier/sulfo-MBS incubation, a 10-ml PD-10 desalting column (Pharmacia Biotech) was equilibrated with 50 ml of Coupling Buffer. To remove excess sulfo-MBS, the carrier/sulfo-MBS solution was loaded onto the column and eluted with 0.5-ml aliquots of Coupling Buffer. The Bradford protein assay (Section 2.5.4.2) was used to identify the peak fractions, which were then pooled. 2 mg of peptide was then dissolved in 100 μl of coupling buffer and the pH was adjusted to 7.5 with 2 M NaOH. The peptide solution was added to the pooled column fraction and incubated for 2 h at room temp to covalently couple the

Table 2.4. Synthetic peptides used for the production and characterisation of phosphorylation state-specific antibodies.

Synthetic Peptide	Sequence (N to C terminus)	Use
β3-S383	CSGIQYRKQSMPK	Characterisation
β3-S383-P	CSGIQYRKQS^{P}MPK	Immunogen and characterisation
β3-S408/S409	CKTHLRRRSSQLK	Characterisation
β3-S408-P	CKTHLRRRS^{P}SQLK	Characterisation
β3-S409-P	CKTHLRRRSS^{P}QLK	Immunogen and characterisation
β3-S408/S409-P	CKTHLRRRS^{P}S^{P}QLK	Characterisation

S^P indicates a phosphorylated serine residue

C highlights the introduction of an N-terminal cysteine residue

cysteine-containing peptide to the cross-linker via a reaction with its maleimide group. The solution was then diluted to a final volume of 1.8 ml with sterile water and five aliquots were prepared as follows: one aliquot of 0.6 ml (for immunisation) and four aliquots of 0.3 ml (for four boosts). Each aliquot was shared between two rabbits. Each rabbit therefore received 300 μg of peptide at the immunisation step and 150 μg of peptide at each boost. The aliquots were stored at -20 °C until required.

2.4.3.3 Production of Antisera

Each peptide antigen was used to raise polyclonal antibodies in two rabbits. This part of the project was contracted out to Cocalico Biologicals Inc, PA, USA. The rabbits were immunised using the standard company protocol, in Freund's Complete Adjuvant. Table 2.5 shows the schedule that was followed for production of the antibodies in rabbits UCL-103 and UCL-104 (for anti-phospho-S383), and rabbits UCL-105 and UCL-106 (for anti-phospho-S409). The methodology used to affinity-purify antibodies in this study is described in Section 2.5.2.

Table 2.5. Timetable for the production of polyclonal phosphorylation state-specific antibodies in rabbits UCL-103, UCL-104, UCL-105 and UCL-106.

Day	Procedure
0	Pre-bleed
0	Initial inoculation
14	Boost
21	Boost
35	Test-bleed 1
49	Boost
56	Test bleed 2
93	Exsanguination

2.5 Biochemistry

2.5.1 Antibodies

A list of all antibodies used in this study is given in Table 2.6.

2.5.2 Affinity-Purification of Antibodies

2.5.2.1 Preparation of Affinity Columns

2.5.2.1.1 Protein A Columns

0.5 g of protein A Sepharose CL-4B (Amersham Biosciences) was re-suspended in 10 ml of deionised water and incubated with rotation for 30 min at 4 °C. The beads were washed twice in Tris Buffered Saline (TBS) (50 mM Tris-HCl and 150 mM NaCl, pH 7.5), with centrifugation at 2000 rpm for 2 min at room temp between washes. After the final wash the beads were re-suspended in 10 ml of TBS and packed into a glass Econo-Column chromatography column (Bio-Rad) to give a 4 to 5 ml bed volume. Once packed, the column was washed with 40 ml of TBS-T (TBS with 0.1% (v/v) Tween-20) and stored at 4 °C until required.

2.5.2.1.2 Phosphopeptide Columns

1 g of activated CH Sepharose 4B (Amersham Biosciences) was re-suspended in 10 ml of 1 mM HCl and incubated with rotation for 10 min at 4 °C. The Sepharose beads were washed twice in 1 mM HCl, with centrifugation, at 2000 rpm for 2 min at 4 °C, between washes. After the final wash, the beads were re-suspended in 10 ml of Coupling Buffer II (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) and incubated, with rotation, for 10 min at 4 °C. Following centrifugation at 2000 rpm for 2 min at 4 °C, the beads were re-suspended in 16 ml Coupling Buffer II. 10 to 15 mg of peptide was then dissolved in 1 ml of water and the pH was adjusted to 7.5 with 2M NaOH. The peptide solution was mixed with 8 ml of Coupling Buffer II and 4 ml of Sepharose beads, and rotated overnight, with rotation, at 4 °C. The following day, the beads were washed twice in Coupling Buffer II, and blocked in 1 M ethanolamine, pH 8, for 1 h. The beads were first washed in Wash Buffer (0.1 M NaOAc and 0.5 M NaCl, pH

Table 2.6. Antibodies used in this study.

Antibody	Description	Source
Mouse anti-CaMKIIα	Monoclonal antibody to recombinant and native phosphorylated and non-phosphorylated CaMKIIα; clone 6G9; 1:1500 dilution	Oncogene Research Products
Rabbit anti-CaMKII-pT286	Polyclonal antibody to CaMKII phosphorylated on T286; 1:2000 dilution	Promega
Rabbit anti-pPKC (pan)	Polyclonal antibody to PKC isoforms (α/βI/βII/δ/ε/η/τ) phosphorylated at a C-terminal residue homologous to S660 of PKC-βII; 1:1000 dilution	Cell Signalling
Anti-μ2	Monoclonal anti-μ2 adaptin antibody; 1:250 dilution	BD Biosciences
UCL-95 and UCL-98	Polyclonal antibodies to residues 345-408 of the $GABA_A$ receptor β3 subunit intracellular domain; used at 0.5 or 1 μg/ml	S.J. Moss
UCL-39 (anti-β3-pS408/S409)	Polyclonal antibody to the $GABA_A$ receptor β3 subunit phosphorylated on S408 and S409; used at 1 μg/ml	S.J. Moss
UCL-103 (anti-β3-pS383)	Polyclonal antibody to the $GABA_A$ receptor β3 subunit phosphorylated on S383; serum was diluted 1:100; affinity-purified used at 1 μg/ml	This Study
UCL-104 (anti-β3-pS383)	Polyclonal antibody to the $GABA_A$ receptor β3 subunit phosphorylated on S383; serum was diluted 1:100	This Study
UCL-105 (anti-β3-pS409)	Polyclonal antibody to the $GABA_A$ receptor β3 subunit phosphorylated on S409; serum was diluted 1:100	This Study
UCL-106 (anti-β3-pS409)	Polyclonal antibody to the $GABA_A$ receptor β3 subunit phosphorylated on S409; serum was diluted 1:100; affinity-purified used at 1 μg/ml	This Study
Rabbit anti-γ2 subunit	Polyclonal antibody to the N-terminus of the $GABA_A$ receptor γ2 subunit	W. Sieghart
Control rabbit IgG	ChromPure rabbit IgG, whole molecule	Jackson Labs
Rabbit anti-mouse IgG	Polyclonal antibody to heavy chains on mouse IgG and light chains common to most mouse immunoglobulins; used at 5 μg/ml	Jackson Labs
Anti-mouse-HRP	Polyclonal antibody to mouse IgG conjugated to horseradish peroxidase; 1:7000 dilution	Jackson Labs
Anti-mouse-HRP (min X)	Peroxidase conjugated affinity purified goat anti-mouse IgG (H&L) (min cross-reactivity); 1:5000 dilution	Rockland Immunochem.
Anti-rabbit-AP	Polyclonal antibody to rabbit IgG conjugated to alkaline phosphatase; 1:1000 dilution	Promega

Antibody	Description	Source
Anti-mouse-AP	Polyclonal antibody to mouse IgG conjugated to alkaline phosphatase; 1:1000 dilution	Promega
[^{125}I]–anti-rabbit antibody	Polyclonal anti-rabbit whole antibody raised in donkey, [^{125}I]-coupled; 1:1000 dilution	Amersham Biosciences

4), and then Coupling Buffer II, pH 8.3. This was repeated three times. The beads were then re-suspended in 10 ml of TBS and packed into a glass Econo-Column chromatography column to give a 4 to 5 ml bed volume. Once packed, the column was washed with 40 ml of TBS-T (Section 2.5.2.1.1) and stored at 4 °C until required.

2.5.2.1.3 GST-β3 (345-408)-Fusion Protein Column

GST-β3 (345-408) fusion proteins were expressed in 2 l of bacterial culture and purified as described in Section 2.2.4. The GST-fusion proteins were dialysed against 2 l of Coupling Buffer II. A CNBr-activated Sepharose 4B column was prepared and stored essentially as described in Section 2.5.2.1.2, except that the Sepharose beads were incubated with GST-β3 (345-408)-fusion proteins instead of peptides.

2.5.2.2 Affinity Purification of Antisera

5 ml of each serum sample (UCL-103, UCL-106, UCL-39, UCL-95 and UCL-98; Table 2.6) was thawed on ice and passed through a 0.45-μm filter. EDTA was added to 5 mM and EGTA to 1 mM. Pepstatin, antipain and leupeptin were each added to 20 μg/ml, and PMSF to 100 μM. The column was washed with 25 ml of TBS-T (Section 2.5.2.1.1), and the serum was passed through the column three times. The column was washed with 30 ml of TBS-T, 30 ml of BBS-T (25 mM Na borate, 3.1 g/l boric acid, 1 M NaCl, pH to 8.3 and 0.1% (v/v) Tween-20), 30 ml of ABS-T (50 mM NaOAc, 1 M NaCl, pH to 5.5 with HCl and 0.1% (v/v) Tween-20) and, finally, 40 ml of TBS. Antibodies were eluted with 0.1 M glycine, pH 2 or 2.5, and immediately neutralised with 1 M Tris base. The peak fractions were pooled and dialysed overnight at 4 °C against 4 l of PBS. Following dialysis, the absorbance at 280 nm was measured using a Bio-Rad SmartSpec 3000 spectrophotometer. The formula for calculating the concentration of antibody, where A_{280} is the absorbance at 280 nm, is

given by

$$1 \text{ mg / ml IgG} = A_{280}\ 1.4 \qquad (2.2)$$

The antibody solution was frozen in aliquots and stored at 4 °C or -20 °C. The column was recycled by several washes, in the following order: 10 ml of TBS, 10 ml of 0.1 M glycine, pH 2 or 2.5, 10 ml of TBS, 10 ml of 4.3 M $MgCl_2$ and finally 10 ml of TBS-T. The column was stored at 4 °C until required.

2.5.3 Preparation of Tissue and Cell Extracts

2.5.3.1 Preparation of Whole Brain Extract

An adult Sprague-Dawley rat (Section 2.4.2.1) brain was homogenised in a 50-cm^3 homogeniser containing 10 ml of Affinity-Purification Buffer (50 mM HEPES, pH 7.6, 1% (v/v) nonidet P-40 (NP-40), 0.5% (w/v) deoxycholate (DOC), 5 mM EGTA, 5 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, pepstatin, antipain and leupeptin (each to 10 μg/ml), 1 mM PMSF and 150 mM NaCl). The homogenate was rotated for 1 h at 4 °C, and transferred to four Beckman polycarbonate centrifuge tubes. Insoluble material was removed by centrifugation in a Beckman optima ultracentrifuge (TLA 110 rotor) at 60,000 rpm for 30 min at 4 °C. The supernatant was retained and kept at 4 °C for the rest of the day. Protein concentration of the extract was determined using the Bradford protein assay (Section 2.5.4.2).

For coimmunoprecipitation (co-IP) studies, a mouse brain was homogenised in a 50-cm^3 homogeniser containing 3 ml of Co-IP Buffer (0.5% (w/v) DOC, 1% (v/v) NP-40, 20 mM HPO_4, pH 8.0, one protease inhibitor cocktail tablet (Roche; Penzberg, Germany) and one phosphatase inhibitor cocktail tablet (Calbiochem) and extracted overnight at 4 °C. Insoluble material was removed by centrifugation in a Beckman optima ultracentrifuge (TLA 110 rotor) at 35,000 rpm for 30 min at 4 °C. The supernatant was retained and kept at 4 °C for the rest of the day. The protein concentration was determined using the Bradford protein assay (Section 2.5.4.2).

2.5.3.2 Preparation of Neuronal Extracts

2.5.3.2.1 Using 2% (w/v) Sodium Dodecyl Sulphate

Dishes were placed on ice and neurons were washed twice with chilled PBS. Cells were lysed in 166 µl of 2% (w/v) Sodium Dodecyl Sulphate (SDS), and the extract was collected using a cell scraper (Corning; NY, USA), and transferred to a microfuge tube. Samples were sonicated for 10 s and boiled for 5 min at 95 °C. The protein concentration of the cell extract was determined using the BCA protein assay (Section 2.5.4.1). Samples were stored at -20 °C until required.

2.5.3.2.2 Using Pre-Labelling/IP Buffer

Dishes were placed on ice and neurons were washed twice with chilled PBS. Cells were lysed in 166 µl PB/SDS Buffer (PBS containing 5 mM EGTA, 5 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, pepstatin, antipain and leupeptin (each to 10 µg/ml), 1 mM PMSF and 1% (w/v) SDS). The extract was collected using a cell scraper and transferred to a 15-ml falcon tube. 1 ml of PB/NP-40 Buffer (PBS containing 5 mM EGTA, 5 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, pepstatin, antipain and leupeptin (each to 10 µg/ml), 1 mM PMSF and 1% (v/v) NP-40) was added and samples were solubilised for 1 h at 4 °C. Samples were either sonicated for 10 s, or passed through a 21-gauge hypodermic needle. Samples were centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatant was retained and kept on ice.

2.5.3.2.3 Using Affinity-Purification Buffer

Dishes were placed on ice and neurons were washed twice with chilled PBS. Cells were then lysed in 500 µl of Affinity-Purification Buffer (Section 2.5.3.1). The extract was collected using a cell scraper and transferred to a 15-ml Falcon tube. Samples were solubilised for 1 h at 4 °C and centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was transferred to a new tube and kept on ice until required, and the protein concentration was determined using the Bradford protein assay (Section 2.5.4.2).

2.5.3.3 Preparation of COS-7 Cell Extracts

COS-7 cells were lysed according to the method described in Section 2.5.3.2.3.

2.5.4 Measurement of Protein Concentration

2.5.4.1 Bicinchoninic Protein Assay

Protein concentration was determined using the Bicinchoninic Protein Assay (BCA) reagents (Pierce). The assay reagent was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. A standard curve was generated by diluting BSA (1 mg/ml; Pierce) in 2 ml of assay reagent to a final concentration of 0.5, 1, 1.5, 2, 2.5, 5, 7.5 and 10 μg/ml, and by adding 15 μl of 2% (w/v) SDS to each tube. 5 μl of unknown sample (containing 2% (w/v) SDS) and 10 μl of 2% (w/v) SDS were added to 2 ml of assay reagent mix. All standards and samples were transferred to a 37 °C water bath for 30 min. The absorbance was measured at 562 nm using a spectrophotometer, and the sample concentration was extrapolated from the standard curve.

2.5.4.2 Bradford Protein Assay

Protein concentration was determined using the Bradford protein assay reagent. A standard curve was generated by diluting BSA (1 mg/ml) in 1 ml of assay reagent (diluted 1:5) to a final concentration of 1, 2, 5 and 10 μg/ml, and measuring the absorbance at 595 nm (A_{595}) with a spectrophotometer. 1 μl of unknown sample was added to 1 ml of the diluted assay reagent, and the A_{595} was measured. Protein concentration was determined by extrapolation from the standard curve.

2.5.5 SDS-Polyacrylamide Gel Electrophoresis

Proteins were resolved by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using Bio-Rad or Invitrogen equipment.

2.5.5.1 Using the Bio-Rad System

Polyacrylamide gels were cast and run using Bio-Rad Mini-PROTEAN®3 or large-format PROTEAN II xi gel apparatus, in accordance with the manufacturer's guidelines. Briefly, the Laemmli resolving gel, which consisted of 10% (w/v)

acrylamide/0.27% (w/v) bisacrylamide (National Diagnostics; Hull, UK), or 12% (w/v) acrylamide/0.32% (w/v) bisacrylamide, and 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate (APS) and 0.04% (v/v) N, N, N', N'-tetramethyl-ethylene diamine (TEMED), was cast between two glass plates and overlaid with water-saturated butanol until gel polymerisation was complete. The Laemmli stacking gel, which consisted of 5.1% (w/v) acrylamide/0.14% (w/v) bisacrylamide, 125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS and 0.1% (v/v) TEMED, was then poured above the resolving gel and left to polymerise after insertion of a comb.

Samples were loaded onto the gel in SDS Loading Buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.0005% bromophenol blue), after being boiled for 5 min at 95 °C. Molecular weight standards (Bio-Rad or Amersham) were also loaded onto each gel. Gels were run in Tris-Glycine Electrophoresis Buffer (25 mM Tris base, 250 mM glycine and 0.1% (w/v) SDS) at approx 70 V (large gels) or 150 V (small gels) until the bromophenol blue dye-front reached the desired position on the gel.

2.5.5.2 Using the Invitrogen System

Novex NuPAGE® 4-12% Bis-Tris pre-cast gels and buffers were used with the XCell *SureLock™* Mini-Cell, in accordance with the manufacturer's guidelines. Samples were prepared and loaded onto the gel, which was run as described in Section 2.5.5.1.

2.5.6 Visualising Proteins in SDS-PAGE Gels by Staining with Brilliant Blue R

To visualise molecular weight markers and other proteins in an SDS-PAGE gel (and to check the loading and degradation of the GST-fusion proteins) the resolving gel was immersed in approx 5 volumes of 0.25% (w/v) Brilliant Blue R in destain solution (30% (v/v) methanol, 10% (v/v) glacial acetic acid) for 15 min. The gel was then rinsed and immersed in destain solution, which was replaced every 15 min until proteins bands became visible. If desired, the gel was also rinsed in 1% (v/v) glycerol for 5 min before drying with a Bio-Rad 583 gel dryer for 1 to 2 h at 80 °C.

2.5.7 Western Blot Analysis

Following separation by SDS-PAGE, protein bands were transferred from the polyacrylamide gel onto a Hybond-C nitrocellulose membrane (Amersham) using Bio-Rad Protean Trans-Blot apparatus, in accordance with the manufacturer's guidelines. Proteins were transferred in Transfer Buffer (48 mM Tris base, 39 mM glycine, 0.037% (w/v) SDS and 20% (v/v) methanol) at 300 mA for 3 h.

The nitrocellulose membrane was rinsed in a solution of 0.1% (w/v) Ponceau-S in 5% (v/v) glacial acetic acid for 2 min at room temp to visualise molecular weight markers and other proteins (and to check the loading and degradation of the GST-fusion proteins). After rinsing with deionised water, the nitrocellulose membrane was incubated with Milk Blocking Buffer (PBS containing 4% (w/v) Marvel milk and 0.1% (v/v) Tween-20) or TBS-T Blocking Buffer (TBS-T (Section 2.5.2.1.1) containing 2 mg/ml BSA), for 1 h at room temp or overnight at 4 °C. The membrane was then incubated with the primary antibody, which was diluted to the appropriate concentration in the blocking buffer, for 1 h at room temp or overnight at 4 °C, with agitation. After three 10 min washes in the blocking buffer, the membrane was incubated with secondary antibody, which was diluted to the appropriate concentration in the blocking buffer, for 1 or 2 h at room temp. The membrane was then washed for 10 min with the blocking buffer followed by two 10-min washes with PBS containing 0.1% (v/v) Tween-20, or TBS-T, as appropriate.

Secondary antibodies were conjugated to horseradish peroxidase (HRP), alkaline phosphatase (AP) or the [^{125}I]-radiolabel (Table 2.6). Those antibodies conjugated to HRP were detected using the Pierce enhanced chemiluminescence (ECL™) system. The membrane was exposed to Kodak X-ray film, and was processed in an RG II Fuji X-ray film processor. Secondary antibodies conjugated to AP were detected by first rinsing the membrane in AP Buffer (100 mM Tris-HCl, 100 mM NaCl and 10 mM $MgCl_2$, pH 9.5), and then in AP Buffer containing 0.35% (v/v) NBT and 0.7% (v/v) BCIP (Promega) until the protein bands were visible. The membrane was rinsed in deionised water to stop the reaction. The [^{125}I]-conjugated secondary antibody was detected by phosphorimaging (Section 2.5.8).

2.5.8 Phosphorimaging

Radioactive protein bands were detected by exposing the membrane to a Bio-Rad phosphorimager screen, which was processed in a Bio-Rad phosphorimager (molecular imager FX). Bio-Rad Quantity One software was used for data capture and quantitation of band intensities.

2.5.9 Dot-Blot Assays

Synthetic peptides (Table 2.4) were dissolved in 50 mM HEPES, pH 7.4, or sterile water to 1 mg/ml and the pH was adjusted to 7.5 with 2 M NaOH, where necessary. The peptides were then serially diluted to give the following concentration range: 100 ng/μl, 50 ng/μl, 25 ng/μl and 5 ng/μl. 1 μl of each peptide solution was spotted onto a PVDF membrane (Immobilon P; Millipore), which had been marked into a grid using a pencil, soaked in methanol and partially dried at room temp. After the spotting, the membrane was soaked in methanol, and washed in water. Western blotting was then performed as previously described in Section 2.5.7, using TBS-T Blocking Buffer, and crude rabbit serum (UCL-103, UCL-104, UCL-105 and UCL-106) diluted 1:100 in TBS-T, and an anti-rabbit-AP secondary antibody (Table 2.6).

2.5.10 Coimmunoprecipitation Assays

A mouse brain extract was prepared as described in Section 2.5.3.1. 10 μg of control rabbit IgG or anti-β3 subunit antibody (Table 2.6), and 50 μl of a 50%-slurry of protein-A beads were added to 0.5 ml of extract, and immunoprecipitations were incubated overnight, with agitation, at 4 °C. The beads were then washed five times in 500 μl of Co-IP Buffer (Section 2.5.3.1), with centrifugation at 4,000 rpm for 2 min at 4 °C in a 5415-R centrifuge, between washes. After the final wash, samples were boiled for 5 min at 95 °C in SDS Loading Buffer, and subjected to SDS-PAGE (Section 2.5.5). Western blotting was then performed (Section 2.5.7) using a primary anti-CaMKIIα antibody and a Rockland secondary anti-mouse-HRP antibody (Table 2.6).

2.5.11 Affinity-Purification ('Pull-Down') Assays

2.5.11.1 Using Tissue or Cell Extracts

A tissue or cell extract was prepared in Affinity-Purification Buffer as described in Sections 2.5.3.2.3 and 2.5.3.3, and the protein concentration was determined as described in Section 2.5.4.2. 0.5 mg or 1 mg of extract was incubated with 25 μg of the appropriate GST-fusion protein (Table 2.2 and Section 2.2.4) and 50 μl of a 50%-slurry of glutathione-agarose beads, for 1 h at 4 °C, with rotation. Samples were then centrifuged at 6,500 rpm for 2 min at 4 °C in a 5415-R centrifuge and washed with 1 ml of Affinity-Purification Buffer. Following two more washes, including one wash in 1 ml of Affinity-Purification Buffer containing 500 mM NaCl, samples were boiled for 5 min at 95 °C in SDS Loading Buffer. Samples were subjected to SDS-PAGE and proteins of interest were detected by Western blotting (Sections 2.5.5 and 2.5.7).

2.5.11.2 Using In Vitro Transcribed/Translated DNA

The plasmid DNA (Table 2.2) of interest was transcribed and translated *in vitro* using the SP6 or T7 Promega TNT® quick-coupled transcription/translation system and easy-tag express [^{35}S]-methionine, in accordance with the manufacturer's guidelines. 5 μl of the *in vitro* transcription/translation reaction was then mixed with 25 μg of the appropriate GST-fusion protein (Table 2.2 and Section 2.2.4) and 50 μl of a 50%-slurry of glutathione-agarose beads, and the total volume was made up to 0.5 ml with *In Vitro* Affinity-Purification Buffer (0.5% (v/v) triton-X-100, 20 mM HEPES, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF and pepstatin, leupeptin and antipain (each at 10 μg/ml). Following incubation for 1 h at 4 °C, with rotation, beads were washed three times with 1 ml of *In Vitro* Affinity-Purification Buffer, with centrifugation at 6,500 rpm for 2 min at 4 °C, between washes. Following the final wash, samples were boiled for 5 min at 95 °C in SDS Loading Buffer, and subjected to SDS-PAGE (Section 2.5.5). GST-fusion proteins and molecular weight markers were visualised (and checked for equal loading where appropriate) by staining with Brilliant Blue R (Section 2.5.6), and [^{35}S]-labelled proteins were detected and quantitated, by phosphorimaging (Section 2.5.8).

2.5.12 Pre-Labelling Assays

Cultured cortical neurons (E17; 6DIV) (Section 2.4.2) were washed twice in 4 ml of

minimum essential medium eagle without phosphate (MEME), supplemented with 2% (v/v) (1X) B27 and 2 mM L-glutamine, and incubated in a further 4 ml of supplemented MEME for 1 h at 37 °C, 5% CO_2 and 100% humidity. 0.5 mCi of [^{33}P]-orthophosphate was then added to the neurons for a further 4 h, and KN93 (4 μM), was also added, where appropriate, during the last 20 min of the 4 h. incubation to enable it to enter neurons and inhibit CaMKII. The neurons were then rinsed twice in 3 ml of HEPES-buffered saline (HBS; 150 mM NaCl, 3 mM KCl, 3 mM calcium chloride, 2 mM magnesium chloride, 5 mM D-glucose, 10 mM HEPES, pH 7.4), and incubated for a further 5 min in HBS, or HBS containing the appropriate treatment: 50 μM muscimol with or without 4 μM KN93.

Following the pre-labelling and neuronal treatments, neurons were lysed in 166 μl of PB/SDS Buffer (Section 2.5.3.2.2) and transferred to a 15-ml falcon. Following addition of 2 ml of PB/NP-40 Buffer (Section 2.5.3.2.2), the cell lysate was rotated for 1 h at 4 °C. The DNA was sheared through a 21-gauge hypodermic needle and samples were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was pre-cleared by incubation with 2 μg of control rabbit IgG and 50 μl of a 50%-slurry of BSA-coated protein-A beads (Amersham) for 30 min at 4 °C, with rotation. Samples were centrifuged at 4,000 rpm for 10 min at 4 °C, and the cleared supernatant was incubated with 2 μg of the appropriate antibody (control rabbit IgG or rabbit anti-β3 subunit) and 50 μl of a 50%-slurry of BSA-coated protein-A beads for 2 h at 4 °C. Following centrifugation at 4,000 rpm for 2 min at 4 °C, the beads were washed in 1 ml of PB/NP-40 Buffer and rotated at 4 °C for 10 min. Following two more washes, samples were boiled for 5 min at 95 °C in SDS Loading Buffer, and subjected to SDS-PAGE (Section 2.5.5). Molecular weight markers were visualised by staining with Brilliant Blue R, and [^{33}P]-labelled proteins were detected by phosphorimaging (Sections 2.5.6 and 2.5.8).

2.5.13 *In Vitro* Kinase Assays

In vitro kinase reactions were set up, on ice, in CaMKII Buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA and 1.5 mM $CaCl_2$) (Grossman *et al.*, 2004) as follows: GST-fusion proteins were mixed with calmodulin (CaM; 10 μg/ml), the PKC

inhibitor, 19-36 (1 μM), the PKA inhibitor, 5-24 (0.1 μM) and CaMKII, which had been purified from brain (1/20 dilution; a generous gift from Howard Schulman Stanford University, CA and Paul Greengard, Rockefeller University, NY (~0.5 mg/ml)). Reactions were incubated at 30 °C for 1 min prior to addition of the ATP mix, which contained ATP (usually at 2 mM) and [γ–^{32}P]–ATP) (usually between 10 and 25% (v/v)). The ATP was added to give a final concentration of 0.2 mM in the kinase assay (except for the phosphatase assays in which it was added to 0.5 mM). Reactions were incubated for the appropriate time at 30 °C and stopped by addition of SDS Loading Buffer. Samples were boiled for 5 min at 95 °C, and proteins were resolved by SDS-PAGE (Section 2.5.5). GST-fusion proteins and molecular weight markers were visualised by staining with Brilliant Blue R (Section 2.5.6), and [^{32}P]-labelled proteins were detected and quantitated by phosphorimaging. The stoichiometry of phosphorylation (mol phosphate mol protein^{-1}) was also calculated, where appropriate (Section 2.5.14).

One variation of this method involved performing kinase assays in the presence of ATP only ('cold' assays) in parallel to assays in the presence of [γ-^{32}P]-ATP and ATP ('hot' assays). The method was essentially as described above, except that before pre-incubation and addition of ATP, 10% (v/v) of each kinase reaction was transferred to a new tube (e.g. 4.5 μl from a total volume of 45 μl) to form a duplicate second set of assays. The first set of reactions was started by addition of ATP (to 200 μM) in a volume that restored the total volume (e.g. 4.5 μl of ATP; the volume previously transferred) and the second set by addition of [γ-^{32}P]-ATP/ATP mix (ATP also to 200 μM, and in a volume that comprised 10% (v/v) of the total volume; e.g. 0.5 μl to give a total volume of 5 μl). Both sets of reactions were incubated in parallel at 30 °C for 15 min. The first set was then immediately transferred to dry ice and stored at -80 °C until required (e.g. for Western blotting with phosphorylation-state specific antibodies). The second set of reactions was stopped upon addition of SDS Loading Buffer and the proteins were resolved by SDS-PAGE. [^{32}P]-labelled proteins were then detected by phosphorimaging after staining all proteins with Brilliant Blue R.

Another variation of this protocol was used in the *In Vitro* Affinity-Purification Kinase Assays (Section 2.5.15). In these, the kinase reactions were performed in the presence of immobilised GST-fusion proteins, which had previously been mixed with a neuronal extract, and were therefore associated with various cellular proteins. The reactions were performed in CaMKII buffer and started upon addition of [γ-^{32}P]-ATP/ATP mix (ATP to 0.2 mM) (calmodulin was not added). The reaction was incubated at 30 °C for 30 min and stopped upon addition of SDS Loading Buffer. Proteins were resolved by SDS-PAGE and then stained with Brilliant Blue R. [^{32}P]-labelled proteins were detected by phosphorimaging.

For *in vitro* kinase assays using purified PKC, two sets of reactions (one 'hot' (25 µl total volume) and one 'cold' (200 µl total volume)) were set up. Each set comprised a phosphorylation reaction and a mock-phosphorylation reaction. Each reaction was set up on ice, in PKC Buffer (20 mM HEPES, pH 7.4, 10 mM MgOAc, 1 mM EGTA and 1.5 mM $CaCl_2$), as follows: GST-γ2S fusion proteins (10 µg: for 'hot' assays; 100 µg: for 'cold' assays), 4 µg/ml DG/50 µg/ml PSEL, and either purified PKC (1/25 dilution; a generous gift from Angus Nairn, Rockefeller University, New York) ('phospho' reaction) or the equivalent volume of sterile water (mock 'phospho') (Picciotto *et al.*, 1992). Reactions were incubated at 30 °C for 1 min before addition of the ATP mix. For one set of GST-γ2S fusion proteins this contained ATP (usually at 2 mM) and [γ–^{32}P]-ATP) (usually between 10 and 25% (v/v)). The ATP was added to give a final concentration of 0.2 mM in the kinase assay. For the other set 'cold' ATP was added to a final assay concentration of 0.2 mM. Reactions were incubated for 60 min at 30 °C. 'Hot' reactions were then stopped by addition of SDS Loading Buffer, and 'cold' reactions by transferring tubes immediately to dry ice, and then to –80 °C. Hot samples were boiled for 5 min at 95 °C, and proteins were resolved by SDS-PAGE (Section 2.5.5). GST-fusion proteins and molecular weight markers were visualised by staining with Brilliant Blue R (Section 2.5.6), and [^{32}P]-labelled proteins were detected and quantitated by phosphorimaging. The stoichiometry of phosphorylation (mol phosphate mol protein^{-1}) was also calculated, where appropriate (Section 2.5.14). The 'cold' samples were then used in affinity-purification assays (see 2.5.11.2).

2.5.14 Calculation of the Stoichiometry of Phosphorylation

The stoichiometry of phosphorylation (mol. phosphate mol. protein^{-1}) was assessed following SDS-PAGE and phosphorimaging by measuring the incorporation of [^{32}P], and normalising to the amount of protein used. To do this, the specific activity of ATP (mol counts^{-1}) was first determined. The ATP mix was diluted 1/10, and two 1-µl spots and two 2-µl spots were pipetted onto a piece of Whatman filter paper (Middlesex, UK). The mean number of counts per µl of ATP mix was then calculated following quantitation of the signals by phosphorimaging (Section 2.5.8), and used to calculate moles of phosphate per count. The number of counts obtained for the GST-fusion protein was then determined by phosphorimaging, and used with the value of the specific activity to calculate the moles of phosphate incorporated into all of the GST-fusion protein present. Secondly, I calculated the total moles of GST-fusion protein present. Finally, I calculated the stoichiometry of phosphorylation (mol phosphate mol protein^{-1}; the moles of phosphate incorporated per mole of protein) by dividing the moles of phosphate incorporated by the total moles of GST-fusion protein present.

2.5.15 *In Vitro* Affinity-Purification Kinase ('Pull-Down'-Kinase) Assays

Cultured cortical neurons (E17; 6DIV) were rinsed twice in HBS (Section 2.5.12), and incubated in 3 ml of HBS, or HBS containing KCl (50 mM), with or without KN93 (4 µM) or EGTA (4 mM) for the appropriate time, at 37 °C. KN93 was added to the neurons, where appropriate, 20 min before treatment to enable it to enter neurons and inhibit CaMKII; EGTA was added 5 min before treatment. Following the treatments, dishes were placed on ice, and neurons were lysed in 500 µl of Affinity-Purification Buffer (Section 2.5.3.1). Following incubation for 1 h at 4 °C, with rotation, the extract was centrifuged at 13,000 rpm for 15 min at 4 °C, and the supernatant was transferred to a new tube. The protein concentration was determined by the Bradford assay (Section 2.5.4.2). 3.5 mg of neuronal extract was mixed with 25 µg of the appropriate GST-fusion protein (Table 2.2 and Section 2.2.4) and 50 µl of a 50%-slurry of glutathione-agarose beads for 1 h at 4 °C, with rotation. Samples were washed three times with 1 ml of Affinity-Purification Buffer, and a further three times in CaMKII Buffer (Section 2.5.13), with centrifugation at 6,500 rpm for 2 min

at 4 °C between washes. Kinase reactions were set up as follows: the beads containing the bound GST-fusion proteins were re-suspended in CaMKII Buffer, on ice. A CaMKII substrate peptide (autocamtide; 100 μM) was added to a fraction of the assay as a further control, but this did not yield reliable data. Reactions were incubated at 30 °C for 1 min before addition of the ATP mix, which contained ATP (usually at 2 mM) and [γ–^{32}P]-ATP (usually between 10 and 25% (v/v)). ATP was added to give a final concentration of 0.2 mM in the kinase assay. Reactions were incubated for 30 min at 30 °C and were stopped by addition of an equal volume of 150 mM phosphoric acid. Samples were centrifuged at 6,500 rpm for 3 min at 4 °C. The excess phosphoric acid was removed and spotted onto P81 Whatmann filter paper, which was then washed 3 times 10 min in phosphoric acid. The [^{32}P]-emission was counted using a scintillation counter. The beads were washed twice in CaMKII Buffer, with centrifugation between washes, as above. Samples were boiled in SDS-PAGE Loading Buffer for 5 min at 95 °C, and subjected to SDS-PAGE (Section 2.5.5). GST-fusion proteins and molecular weight markers were visualised with Brilliant Blue R, and [^{32}P]-labelled proteins were detected by phosphorimaging (Sections 2.5.6 and 2.5.8).

2.5.16 *In Vitro* Dephosphorylation Assays

2.5.16.1 Preparative Phosphorylation of Substrates

2.5.16.1.1 Preparative Phosphorylation of GST-β3 Fusion Proteins by CaMKII

The GST-fusion proteins, GST-β3, GST-β3-S383A and GST-β3-S409A were expressed and purified as described in Table 2.2 and Section 2.2.4. Following dialysis against 4 l of PBS, the GST-fusion proteins were concentrated using Amicon Ultra-15 PLGC centrifugal filter units (Millipore; Billerica, MA, USA). 330 μg of each GST-fusion protein was then phosphorylated *in vitro* by purified CaMKII (1/20 dilution) in the presence of [γ-^{32}P]-ATP, as described in Section 2.5.13. The reaction was stopped after 30 min at 30 °C, by using a Sephadex G-50 NICK column (Amersham Biosciences) to separate phosphorylated GST-fusion proteins from ATP and [γ-^{32}P]-ATP. [^{32}P]-labelled phospho-GST-fusion proteins were stored in 50%

(v/v) glycerol at -20 °C until required.

2.5.16.1.2 Preparative Phosphorylation of Phosphorylase b by Phosphorylase Kinase

Phosphorylase b (Calzyme, CA, USA) was phosphorylated *in vitro* in a total reaction volume of 2 ml. The reaction was set up as follows: 32 mg of phosphorylase b, 2 mg of phosphorylase kinase, 200 μM ATP and 100 μl of [γ-^{32}P]-ATP in 1X Reaction Buffer (100 mM Tris, pH 8.2, 100 mM Na-Glycerol-1-P, 0.1 mM $CaCl_2$ and 10 mM magnesium acetate). The reaction was incubated for 1 h at 30 °C, and was stopped by desalting on a PD-10 column. An equal volume of 90% (w/v) saturated ammonium sulphate was added to the reaction mix. The solution was centrifuged at 13,000 rpm for 10 min at room temp, and the pellet was washed with 45% (w/v) ammonium sulphate. After re-centrifuging, the pellet was re-suspended in 100 μl of Buffer I (50 mM Tris-HCl, pH 7, 0.1 mM EGTA, 1 mM DTT and 10% (v/v) glycerol) and desalted into the same buffer using a Sephadex G-50 NICK column. The solution was incubated overnight at 4 °C to allow for re-crystallisation. The crystals were centrifuged at 13,000 rpm for 15 min and re-suspended in Buffer I. The supernatant was concentrated by dialysing overnight in 10 mM Tris-HCl, pH 7, and 0.1% (v/v) 2-mercaptoethanol, at 4 °C. The contents of the dialysis cassette were transferred to a new tube and incubated on ice for 30 min. The crystals were centrifuged and re-suspended as before, and the solution was pooled with that previously obtained. [^{32}P]-labelled phosphorylase a was stored at 4 °C until required.

2.5.16.1.3 Preparative Phosphorylation of DARRP-32 by PKA

Tom McAvoy performed the preparative phosphorylation of DARRP-32. Briefly, DARRP-32 was phosphorylated at T34 *in vitro* by the catalytic subunit of PKA in the presence of [γ-^{32}P]-ATP (Hemmings *et al.*, 1984). [^{32}P]-labelled DARRP-32-T34 was separated from ATP and [γ-^{32}P]-ATP by using a Sephadex G-50 NICK column. [^{32}P]-labelled phospho-DARRP-32-T34 was stored in 50% (v/v) glycerol at -20 °C until required.

2.5.16.1.4 Preparative Phosphorylation of Casein by PKA

Hydrolysed casein was phosphorylated *in vitro* in a total reaction volume of 100 µl. The reaction was set up on ice, as follows: 5 µl of 5% (w/v) casein, 500 µM ATP, 10 µl of purified PKA catalytic subunit and 20 µl of [γ-^{32}P]-ATP in 1X PKA Buffer (50 mM HEPES, pH 7.4, 10 mM magnesium acetate and 1 mM EGTA). The reaction was stopped after 1 h at 30 °C, by using a Sephadex G-50 NICK column to separate phosphorylated casein from ATP and [γ-^{32}P]-ATP. [^{32}P]-labelled phospho-casein was stored in 50% (v/v) glycerol at -20 °C until required.

2.5.16.2 Determination of Initial Rate Conditions

All protein phosphatases used in this study were generous gifts from Paul Greengard. Protein phosphatases 1 (PP1) and 2A (PP2A) were purified from rabbit skeletal muscle and human red blood cells, respectively, and were purchased from Upstate (Lake Placid, NY, USA). Protein phosphatase 2B (PP2B; calcineurin) was purified from rat brain (Sihra *et al.*, 1995). I also used a recombinant α isoform of protein phosphatase 2C (PP2Cα) that was cloned from brain (Flajolet *et al.*, 2003).

PP1, PP2A, PP2B and PP2C phosphatase activities were assayed using the reference substrates, [^{32}P]-phosphorylase a (PP1 and PP2A), [^{32}P]-DARRP-32 and [^{32}P]-casein, respectively, in the appropriate assay buffer. The PP1 and PP2A Reaction Buffer consisted of 0.01% (v/v) Brij-35, 0.1 mM EGTA, 5 mM caffeine, 50 mM Tris, pH 7.5, 0.1% (v/v) 2-mercaptoethanol and BSA (1 mg/ml). The PP2B Reaction Buffer consisted of 1 mM $CaCl_2$, 1 µM CaM, 6 mM $MgCl_2$, 100 mM KCl, 20 mM Tris, pH 7.5, 1 mM ATP, 0.1% (v/v) 2-mercaptoethanol and BSA (1 mg/ml). The PP2C Reaction Buffer consisted of 50 mM Tris, pH 8, 40 mM $MgCl_2$, 150 mM NaCl, 5 mM DTT and BSA (10 mg/ml).

Dephosphorylation assays were performed in duplicate in at least three individual experiments (see Jovanovic *et al.*, 2001). Each protein phosphatase was diluted as desired in the appropriate reaction buffer, on ice. 10 µl of diluted protein phosphatase (or of the reaction buffer as a blank) was transferred to a tube containing 10 µl of the appropriate reaction buffer, on ice. Each tube was placed in a water bath at 30 °C for 5 min, before the reactions were initiated by addition of 10 µl of [^{32}P]-labelled

reference substrate (phosphorylase a, DARRP-32 and casein; ~1.5 μM; to give a final concentration of ~0.5 μM). The reactions proceeded for the desired time at 30 °C, and were stopped by addition of 200 μl of 20% (w/v) trichloroacetic acid (TCA). The tubes were vortexed briefly and centrifuged at 13,000 rpm for 3 min at room temp. 100 μl of each supernatant was transferred to a new tube, and the [^{32}P]-emission was measured for 1 min using a liquid scintillation counter and Cerenkov programme to determine the number of sample counts per min (cpm). 10 μl of the [^{32}P]-reference substrate (~1.5 μM) was added to 20 μl of reaction buffer and counted with the supernatants to determine total cpm. The formula for calculating percent phosphate removed, which should be 15% to 25% of the total counts, is given by

$$\% \text{ phosphate removed} = \frac{\text{sample cpm - blank cpm}}{\text{total cpm}} \times 100 \qquad (2.3)$$

2.5.16.3 In Vitro Dephosphorylation Assays of Unknown Substrates

Dephosphorylation assays were performed in duplicate in at least three separate experiments, essentially as described in Section 2.5.16.2. The [^{32}P]-labelled reference substrate (~0.5 μM) and the [^{32}P]-labelled GST-fusion protein unknown substrate (~0.5 μM) were assayed under initial rate conditions (the release of phosphate is linear with respect to enzyme concentration and time, and was 15-25% of the [^{32}P]-phosphate incorporated into the substrate (Jovanovic *et al.*, 2001)) (Section 2.5.16.2). Analysis of dephosphorylation was performed using the formula in Section 2.5.16.2.

2.6 Bioinformatics

Searches were performed using the NCBI database (www.ncbi.nlm.nih.gov). Multiple sequence alignments were constructed using ClustalW (Higgins, 1994) and manual editing with The Jalview Java Alignment Editor (Clamp *et al.*, 2004).

CHAPTER 3

$GABA_A$ Receptors Interact with CaMKII

3.1 Background

In Chapter 1, I discussed the known cellular mechanisms that are involved in the trafficking, sub-cellular localisation and functional modulation of $GABA_A$ receptors. I highlighted the importance of these processes in regulating the efficacy of inhibitory neurotransmission and neuronal excitability, and drew particular attention to the crucial role of phosphorylation-dependent modification. Moreover, I described the known associations between protein kinases and $GABA_A$ receptors, and related these interactions, where possible, to modifications of the phosphorylation state and function of these receptors. This suggested that the differential targeting of protein kinases to neuronal $GABA_A$ receptors is a key step during phosphorylation-dependent functional modulation. It is therefore fundamental to our understanding of GABAergic neurotransmission to understand how kinases are targeted to $GABA_A$ receptor scaffolds.

The results of electrophysiological studies based on the modulation of postsynaptic calcium/CaM signalling cascades and intracellular application of CaMKII suggest that CaMKII is involved in modulating GABAergic neurotransmission (Kano *et al.*, 1996; Kawaguchi and Hirano, 2002; Wei *et al.*, 2004; Wang *et al.*, 1995; Aguayo *et al.*, 1998; see Section 1.8 for an overview of the structure and regulation of CaMKII). In cortical neurons, an increase in intracellular calcium concentration has been shown to cause transient augmentation of the GABA current, and inhibitors of both CaM and CaMKII have been shown to block this effect (Aguayo *et al.*, 1998). In hippocampal pyramidal neurons, postsynaptic activation of IP_3 receptors and induction of calcium release results in an enhancement of $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) (Wei *et al.*, 2004). The calcium chelator, BAPTA, and a CaM-binding peptide have each been shown to block this effect (Wei *et al.*, 2004). Similarly, the postsynaptic application of calcium/CaM leads to an enhancement of both evoked and spontaneous $GABA_A$ receptor-mediated IPSCs (Wei *et al.*, 2004). This is attenuated by the autoinhibitory peptide of CaM-dependent protein kinases, CaM-KII (281-301) (Wei *et al.*, 2004). Intracellular application of the alpha subunit of CaMKII has been shown to potentiate GABA-induced currents in spinal dorsal horn neurons, and evoked IPSPs in hippocampal CA1 neurons (Wang *et al.*, 1995).

Moreover, in cerebellar Purkinje neurons, intracellular application of purified, active CaMKII has been shown to enhance the amplitude of both GABA-mediated currents and spontaneous IPSCs (Kano *et al.*, 1996). In cerebellar Purkinje neurons, GABA-mediated inhibitory synaptic currents undergo a long lasting "rebound potentiation" following activation of an excitatory synaptic input and elevation of the intracellular calcium concentration (Kano *et al.*, 1992). Interestingly, a CaMKII inhibitor, KN62, has been shown to block the induction of "rebound potentiation" (Kano *et al.*, 1996). Furthermore, CaMKII may be directly involved in the induction of this potentiation, as a regulatory calcineurin/PKA/DARRP-32/PP1 signalling cascade has been located upstream of CaMKII activity (Kawaguchi and Hirano, 2002).

The above evidence suggests that CaMKII potentiates the activity of $GABA_A$ receptors in neurons. However, the molecular mechanism of such regulation remains to be elucidated. It is possible that CaMKII lies upstream of other cellular proteins that modify $GABA_A$ receptor function, or that CaMKII modifies receptor activity directly. The latter process seems to be a likely candidate since CaMKII is one of a number of protein kinases so far identified, which are capable of phosphorylating $GABA_A$ receptors directly (McDonald and Moss, 1994, 1997; Moss and Smart, 2001; Kittler and Moss, 2003; Section 1.7.2.1; Table 1.1). *In vitro* kinase assays have shown that CaMKII directly phosphorylates specific residues within the major intracellular domains of $GABA_A$ receptor β and $\gamma 2$ subunits (McDonald and Moss, 1994, 1997; Table 1.1). These include S384 and S409 in the $\beta 1$ subunit (McDonald and Moss, 1994), S410 in the $\beta 2$ subunit, and S383 and S409 in the $\beta 3$ subunit (McDonald and Moss, 1997). The $\gamma 2$ subunit sites include S348 and T350, as well as S343 in $\gamma 2L$ only (McDonald and Moss, 1994; Machu *et al.*, 1993).

Although $GABA_A$ receptors are *in vitro* substrates of CaMKII, a physical relationship between CaMKII and $GABA_A$ receptors remains to be determined. Several processes could regulate such an interaction, including kinase activation, sub-cellular targeting by anchoring proteins and the phosphorylation state of receptor subunits. Interestingly, autophosphorylation of CaMKII is known to play a critical role in modulating the sub-cellular localisation of this kinase. For example, phosphorylation

of T286 is implicated in promoting the association of CaMKII with the PSD (McNeill and Colbran, 1995; Shen and Meyer, 1999; Shen *et al.*, 2000), while phosphorylation of T305/306 is thought to destabilise this interaction (Shen *et al.*, 2000; Elgersma *et al.*, 2002; Weeber *et al.*, 2003). The phosphorylation state of CaMKII has also been shown to influence its binding to a number of postsynaptic proteins (McNeill and Colbran, 1995; Merrill *et al.*, 2005). For example, autophosphorylation of T286 is necessary for the association of CaMKII with the NR1 subunit of the NMDA receptor (Leonard *et al.*, 1999, 2002), and potentiates binding to the NR2A (Gardoni *et al.*, 1999, 2001) and NR2B receptor subunits (Bayer *et al.*, 2001). The spatial distribution of CaMKII in neurons can also be governed by the activity of PKC (Fong *et al.*, 2002).

CaMKII may stably bind and remain associated with the $GABA_A$ receptor to ensure that the desired stoichiometry of phosphorylation is achieved. However, CaMKII may also form a transient, unstable association with the receptor complex. In this chapter, I have taken a biochemical-based approach using coimmunoprecipitation and affinity-purification 'GST-pull-down' assays, to investigate how CaMKII is recruited to the $GABA_A$ receptor to mediate phosphorylation. I set the following aims: (i) to determine whether the $GABA_A$ receptor forms a native complex with CaMKII in brain, and if so, (ii) to determine whether the interaction between the $GABA_A$ receptor and CaMKII is direct, (iii) to identify specific regions in both $GABA_A$ receptor subunits and CaMKII isozymes, which are involved in binding, and (iv) to investigate how the interaction between the $GABA_A$ receptor and CaMKII is regulated. This approach has led to the discovery that CaMKII forms a stable phosphorylation-dependent interaction with the $GABA_A$ receptor, and that CaMKII docks to a site in the N-terminus of the major intracellular domain of the receptor β3 subunit. I propose that this interaction underlies the ability to differentially target CaMKII to $GABA_A$ receptors, and that this is a key step in the regulation of $GABA_A$ receptor function in neurons.

3.2 Results

An overview of the methodology used in this chapter can be seen in Figure 3.1.

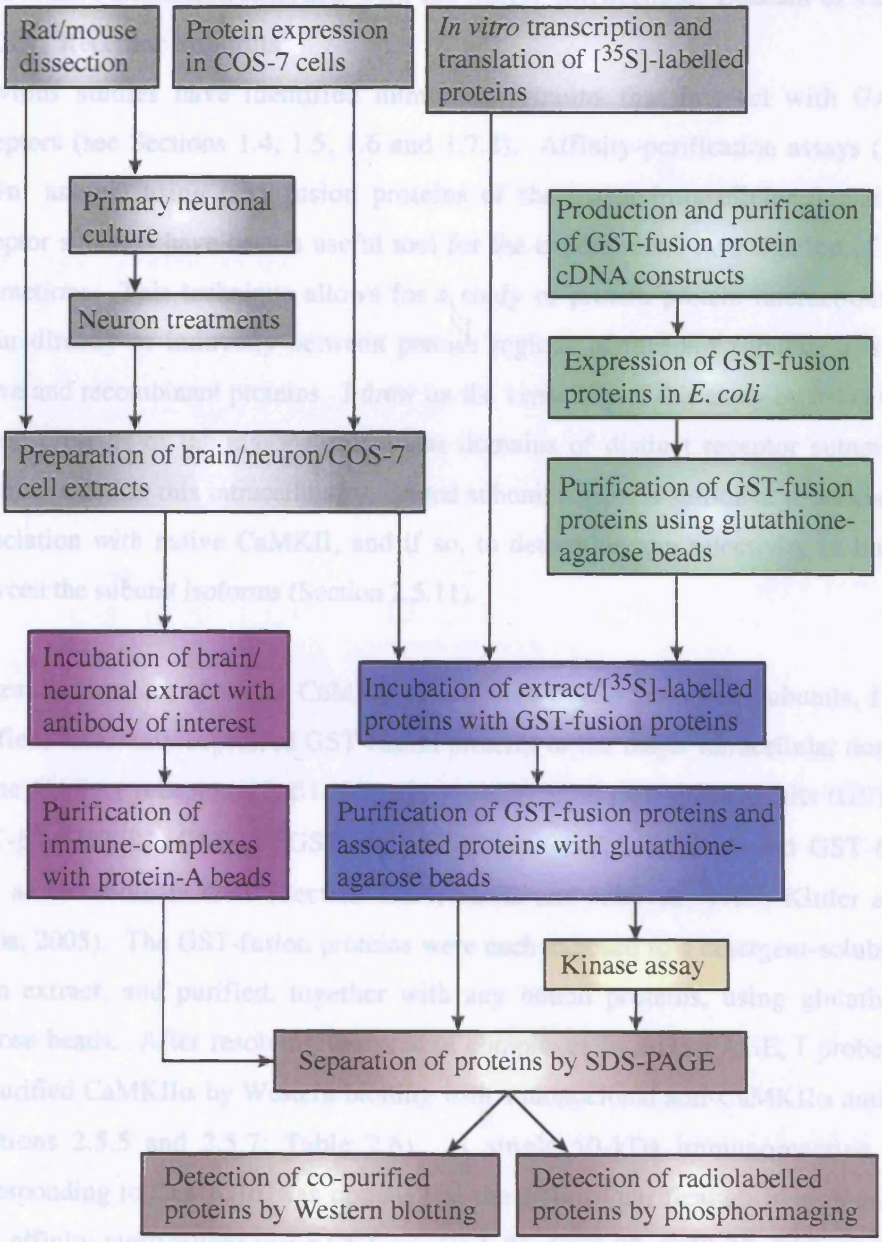


Figure 3.1. General overview of the methodologies employed to investigate $GABA_A$ receptor phosphorylation and receptor-protein interactions. Boxes shaded in grey describe the preparation of tissue and cell extracts, and *in vitro* transcribed and translated radiolabelled proteins. Green boxes describe the production and purification of GST-fusion proteins. Pink boxes describe immunoprecipitation assays and blue boxes describe affinity-purification ('GST-pull-down') assays. The yellow box represents *in vitro* kinase assays and the taupe boxes describe the techniques used to visualise proteins.

3.2.1 Native CaMKIIα Interacts with the Major Intracellular Domain of Various $GABA_A$ Receptor Subunits

Previous studies have identified numerous proteins that interact with $GABA_A$ receptors (see Sections 1.4, 1.5, 1.6 and 1.7.2). Affinity-purification assays ('pull-down' assays) using GST-fusion proteins of the major intracellular domains of receptor subunits have been a useful tool for the experimental investigation of these interactions. This technique allows for a study of protein-protein interactions that occur directly or indirectly between precise regions of receptor subunits and both native and recombinant proteins. I drew on the versatility of this assay by using GST-fusion proteins of the major intracellular domains of distinct receptor subunits to examine whether this intracellularly located subunit region is sufficient to mediate an association with native CaMKII, and if so, to determine any selectivity in binding between the subunit isoforms (Section 2.5.11).

To test for binding of native CaMKII to different $GABA_A$ receptor subunits, I used purified, bacterially expressed GST-fusion proteins of the major intracellular domains of the $GABA_A$ receptor α1, β1, β2, β3, γ1, γ2S, γ2L, γ3 and δ subunits (GST-α1, GST-β1, GST-β2, GST-β3, GST-γ1, GST-γ2S, GST-γ2L, GST-γ3 and GST-δ), as well as recombinant GST (Section 2.2.4; Smith and Johnson, 1988; Kittler *et al.*, 2000a, 2005). The GST-fusion proteins were each exposed to a detergent-solubilised brain extract, and purified, together with any bound proteins, using glutathione-agarose beads. After resolving the protein complexes by SDS-PAGE, I probed for co-purified CaMKIIα by Western blotting with a monoclonal anti-CaMKIIα antibody (Sections 2.5.5 and 2.5.7; Table 2.6). A single 50-kDa immunoreactive band corresponding to CaMKIIα was obtained in the affinity-purification input lane, and from affinity-purifications using GST-α1, GST-β1, GST-β2, GST-β3, GST-γ1, GST-γ2S, GST-γ2L, GST-γ3 and GST-δ, but not GST alone (Fig. 3.2A). These results demonstrated that the major intracellular domains of various $GABA_A$ receptor subunits (α1, β1-3, γ1-3 and δ) mediated either direct or indirect binding to native CaMKIIα. This suggested that the major intracellular domain is sufficient for the interaction of CaMKII with $GABA_A$ receptor subunits, and that neuronal CaMKIIα binds non-selectively to the major intracellular domains of various $GABA_A$ receptor

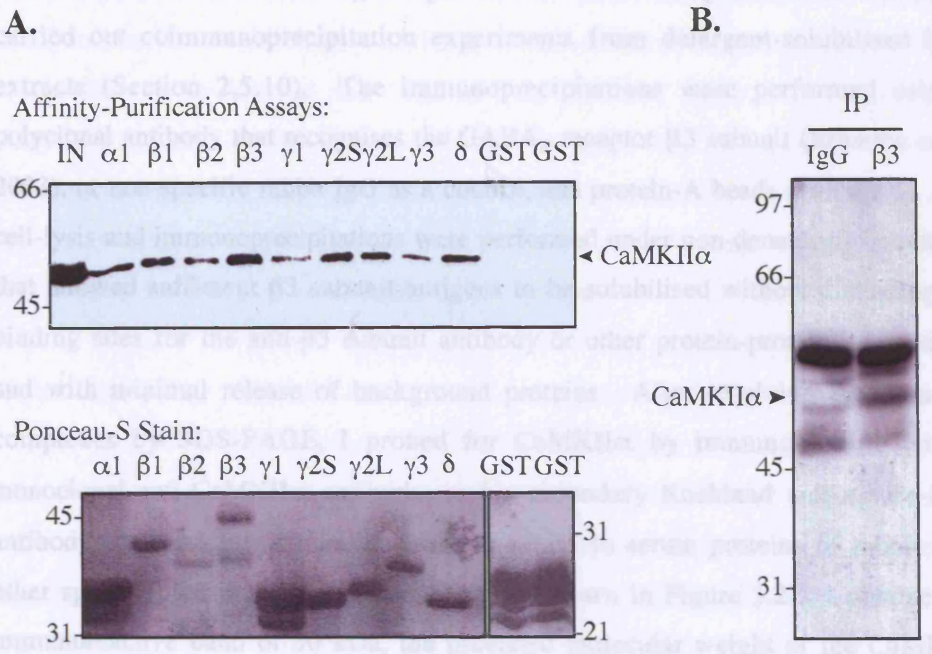


Figure 3.2. CaMKIIα physically interacts with $GABA_A$ receptors *in vitro* and *in vivo*. **A.** Neuronal CaMKIIα binds to the major intracellular domain of various $GABA_A$ receptor subunits. **Upper panel:** affinity-purification assays were performed using 20 μg of GST-fusion proteins of the major intracellular domain of receptor α1, β1, β2, β3, γ1, γ2S, γ2L, γ3 and δ subunits, as well as GST alone. The GST-fusion proteins were exposed to a detergent-solubilised brain extract, and precipitated using glutathione-agarose beads. The protein complexes were then resolved by SDS-PAGE and probed for CaMKIIα by immunoblotting with a monoclonal anti-CaMKIIα antibody (CaMKIIα), and a Jackson (in 'A.') or Rockland (in 'B.') anti-mouse-HRP antibody for detection. 'IN' represents 1% (v/v) of the extract used in each affinity-purification assay. **Lower panel:** ponceau-S stain of the GST-fusion proteins in 'A.'. **B.** CaMKIIα co-immunoprecipitates with $GABA_A$ receptors containing the β3 subunit from brain. Immunoprecipitations were performed from a detergent-solubilised mouse brain extract using control rabbit non-immune IgG or a rabbit polyclonal anti-β3 subunit antibody, and protein-A beads. Precipitated proteins were resolved by SDS-PAGE and probed for CaMKIIα as in 'A.'.

subunits, *in vitro*.

3.2.2 CaMKIIα Forms a Native Complex with $GABA_A$ Receptors in Brain

To determine whether $GABA_A$ receptors form native complexes with CaMKII, I carried out coimmunoprecipitation experiments from detergent-solubilised brain extracts (Section 2.5.10). The immunoprecipitations were performed using a polyclonal antibody that recognises the $GABA_A$ receptor β3 subunit (Brandon *et al.*, 2003), or non-specific rabbit IgG as a control, and protein-A beads (Table 2.6). The cell-lysis and immunoprecipitations were performed under non-denaturing conditions that allowed sufficient β3 subunit antigens to be solubilised without disrupting the binding sites for the anti-β3 subunit antibody or other protein-protein interactions, and with minimal release of background proteins. After resolving the immune-complexes by SDS-PAGE, I probed for CaMKIIα by immunoblotting with a monoclonal anti-CaMKIIα antibody, and a secondary Rockland anti-mouse-HRP antibody (that exhibits minimum cross-reactivity to serum proteins of rabbit (and other species)) for detection (Table 2.6). As shown in Figure 3.2B, I obtained an immunoreactive band of 50 kDa, the predicted molecular weight of the CaMKIIα isozyme, from immunoprecipitations performed with the anti-β3 subunit antibody, but not control IgG. These data demonstrated that CaMKIIα was coimmunoprecipitated with the anti-β3 subunit antibody via a direct or indirect association with the β3 subunit. This suggested that CaMKIIα-containing holoenzymes and β3-subunit-containing $GABA_A$ receptors are both present in a protein complex in brain.

3.2.3 The Interaction of Recombinant CaMKIIα with the Major Intracellular Domains of $GABA_A$ Receptor β Subunits is Phospho-Dependent

3.2.3.1 The Major Intracellular Domains of $GABA_A$ Receptor β Subunits Bind Selectively to the Constitutively Active CaMKIIα-T286D Mutant

I was next interested in examining the effect of phosphorylation of CaMKIIα at T286 on the binding of the kinase to $GABA_A$ receptors, because of its critical role in the targeting of CaMKII to specific sub-cellular locations (Section 3.1). To address this

issue, I performed affinity-purification assays from extracts of COS-7 cells transiently transfected with wild type CaMKIIα (CaMKIIα-WT) or mutant CaMKIIα isozymes (Section 2.4.1 and Table 2.2). I utilised a CaMKIIα-T286D mutant, which mimics autophosphorylation at T286 thus rendering the kinase constitutively active, and an autophosphorylation-deficient CaMKIIα-T286A mutant, which is predominantly in the inactive conformational state (Section 1.8). By using expression of recombinant CaMKIIα in COS-7 cells, I was able to investigate CaMKII proteins that were permanently modified in a cellular environment. The T286D mutation also enabled me to investigate the activated form of the kinase without having to trigger intracellular changes in the concentration of calcium.

To test the binding of the different CaMKIIα constructs to $GABA_A$ receptor subunits, I used purified, bacterially expressed GST-fusion proteins of the major intracellular domains of the $GABA_A$ receptor β subunits, and recombinant GST. The β subunits were chosen for further investigation because they are essential components of all functional GABA-gated receptors (Connolly *et al.*, 1996a,b; Gorrie *et al.*, 1997; Perez-Velazquez and Angelides, 1993), and because they are currently the most extensively characterised receptor subunit with regard to protein-protein interactions and phosphorylation (Moss and Smart, 2001; Kittler and Moss, 2003). To perform the assays, the GST-fusion proteins were each exposed to each of the COS-7 cell extracts, and purified, together with any bound proteins, with glutathione-agarose beads. After resolving the protein complexes by SDS-PAGE, I probed for co-purified CaMKIIα by Western blotting with a monoclonal anti-CaMKIIα antibody.

I obtained a single ~50-kDa immunoreactive band corresponding to recombinant CaMKIIα in the input lanes of affinity-purification assays from CaMKIIα-WT-, CaMKIIα-T286D- and CaMKIIα-T286A-transfected cells, but not untransfected cells (Fig. 3.3, left hand side panels). Whereas CaMKIIα-WT and CaMKIIα-T286A both migrated during SDS-PAGE to give 50-kDa bands, I observed a small upward band-shift with CaMKIIα-T286D to ~51 kDa (Fig. 3.3). Nevertheless, these data showed that CaMKIIα was not endogenously expressed at a detectable level in COS-7 cells, and that the wild type and mutant CaMKIIα isozymes were expressed following

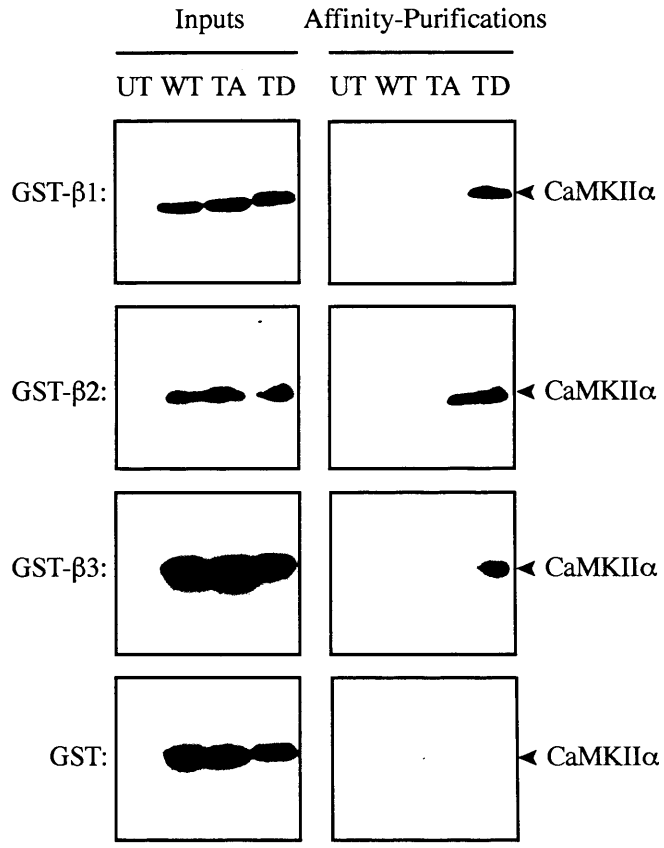


Figure 3.3. The major intracellular domains of the $GABA_A$ receptor β subunits bind selectively to a constitutively active CaMKIIα-T286D mutant. COS-7 cells were mock-transfected (UT) or transiently transfected with wild type CaMKIIα (WT), autophosphorylation-deficient CaMKIIα-T286A (TA) or constitutively active CaMKIIα-T286D (TD), and lysed 24 h later under non-denaturing conditions. Affinity-purification assays were performed using 20 μg each of GST-β1, GST-β2 and GST-β3, as well as GST alone. Each of the GST-fusion proteins was exposed to each of the COS-7 cell extracts, and precipitated using glutathione-agarose beads. Proteins were then resolved by SDS-PAGE and probed for CaMKIIα by immunoblotting with a monoclonal anti-CaMKIIα antibody (CaMKIIα) and an anti-mouse-HRP antibody for detection. 'Inputs' represent 10% (v/v) of the COS-7 cell extract used in each respective affinity-purification assay.

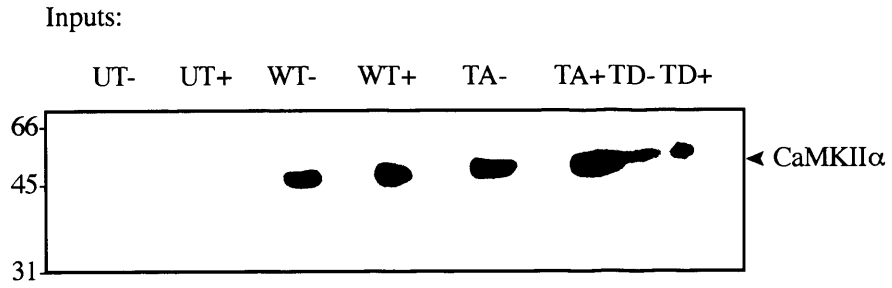
transfection. Furthermore, it showed that all the expressed proteins remained stable throughout the assay. I also obtained ~51-kDa bands from affinity-purification assays using GST-β1, GST-β2 and GST-β3 from extracts of COS-7 cells transiently transfected with CaMKIIα-T286D, but not CaMKIIα-T286A, CaMKIIα-WT or untransfected cells (Fig. 3.3 right hand side panels). I did not obtain bands with control GST from any affinity-purification assays (Fig. 3.3 right hand side panels). These results demonstrated that constitutively active CaMKIIα, but not wild type or autophosphorylation-deficient CaMKIIα interacted with the major intracellular domains of the $GABA_A$ receptor β1, β2 and β3 subunits. This suggested that CaMKIIα binds directly or indirectly to the major intracellular domains of the $GABA_A$ receptor β subunits only when phosphorylated at T286, and that formation of CaMKII-$GABA_A$ receptor complexes *in vivo* may be dependent upon neuronal activity and kinase autophosphorylation.

3.2.3.2 Binding of CaMKIIα-T286D to the Major Intracellular Domain of the β3 Subunit Requires EGTA

I next investigated whether EGTA affected the physical relationship between the wild type and mutant CaMKIIα isozymes and the intracellular domain of the $GABA_A$ receptor β3 subunit. I performed affinity-purification assays from extracts of COS-7 cells transiently transfected with CaMKIIα-WT, CaMKIIα-T286A or CaMKIIα-T286D, using GST-β3. The protocol was identical to that described in Section 3.2.3.1, except that the assays were performed in duplicate, with EGTA either absent from or present in the extraction/assay buffer. After resolving the protein complexes by SDS-PAGE, I probed for co-purified CaMKIIα by Western blotting with a monoclonal anti-CaMKIIα antibody.

I obtained a single ~50-kDa band corresponding to recombinant CaMKIIα in all of the input lanes of affinity-purification assays from CaMKIIα-WT-, CaMKIIα-T286A- and CaMKIIα-T286D-transfected cells, but not untransfected cells (Fig. 3.4A). Once again, I observed immunoreactive bands for CaMKIIα-WT and CaMKIIα-T286A at 50-kDa, and at ~51 kDa for CaMKIIα-T286D (Fig. 3.4). These data again showed that CaMKIIα was not endogenously expressed to a detectable

A.



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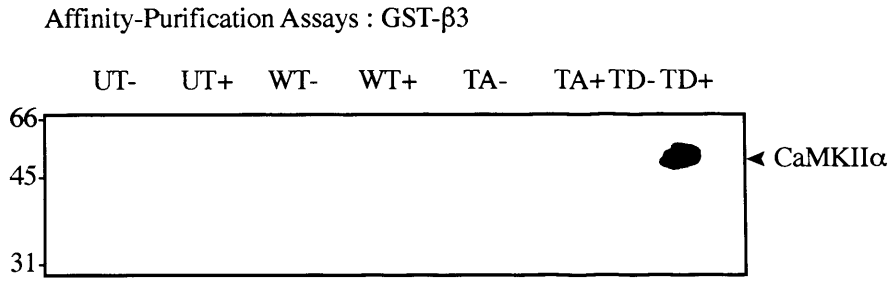


Figure 3.4. Binding of CaMKIIα-T286D to the major intracellular domain of the β3 subunit *in vitro* requires EGTA. COS-7 cells were mock-transfected (UT) or transiently transfected with wild type CaMKIIα (WT), CaMKIIα-T286A (TA) or CaMKIIα-T286D (TD), and lysed 24 h later under non-denaturing conditions in a buffer either with (+) or without (-) 2 mM EGTA. Affinity-purification assays were performed using 20 μg of GST-β3. Each of the GST-fusion proteins was exposed to each of the COS-7 cell extracts, and precipitated using glutathione-agarose beads. Proteins were then resolved by SDS-PAGE and probed for CaMKIIα by immunoblotting with a monoclonal anti-CaMKIIα antibody (CaMKIIα) and an anti-mouse-HRP antibody for detection. **A.** Analysis of assay inputs, which represent 10% (v/v) of the COS-7 cell extract used in each respective affinity-purification assay. **B.** Analysis of the affinity-purification assays.

level in COS-7 cells, and that the wild type and mutant CaMKIIα isozymes were expressed following transfection. Furthermore, it showed that all the expressed proteins remained stable throughout the assay. I also obtained an ~51-kDa immunoreactive band from affinity-purification assays from extracts of COS-7 cells transiently transfected with CaMKIIα-T286D, in which EGTA was present in the extraction/assay buffer (Fig. 3.4B). A band was not obtained in the assays from extracts of COS-7 cells transiently transfected with CaMKIIα-T286D, in which EGTA was absent from the buffer, or from cells transfected with CaMKIIα-T286A or CaMKIIα-WT, or untransfected cells, in the absence or presence of EGTA (Fig. 3.4B). These assays demonstrated that the association of the constitutively active form of CaMKIIα, CaMKIIα-T286D, with the major intracellular domain of the $GABA_A$ receptor β3 subunit was dependent upon EGTA, but that the binding status of wild type and autophosphorylation-deficient CaMKIIα (CaMKIIα-WT and CaMKIIα-T286A, respectively) was unchanged by EGTA. This suggested that, *in vitro*, EGTA either disinhibits or promotes the interaction of CaMKIIα-T286D with the major intracellular domain of the $GABA_A$ receptor β3 subunit.

3.2.4 Consensus *In Vivo* Phosphorylation Sites in the Major Intracellular Domain of the β3 Subunit are not Critical for the Interaction with Native CaMKIIα

My previous findings suggested that kinase phosphorylation at T286 mediated binding of CaMKIIα to the major intracellular domain of the $GABA_A$ receptor β subunits (Figs. 3.3 and 3.4). I was therefore interested in examining whether phosphorylation of $GABA_A$ receptor β subunits might also be involved in mediating this interaction.

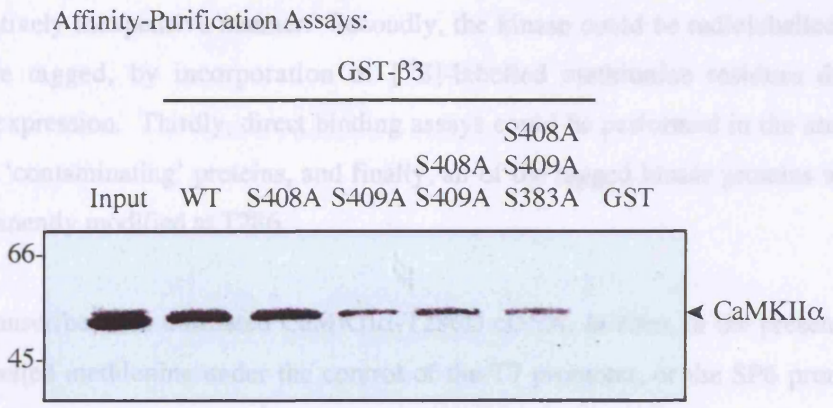
To investigate the role of $GABA_A$ receptor phosphorylation in CaMKIIα binding, I employed affinity-purification assays, and used purified, bacterially expressed wild type and mutant GST-fusion proteins of the major intracellular domain of the β3 subunit, and recombinant GST. The mutant GST-fusion proteins contained serine-to-alanine point mutations of consensus, *in vivo* CaMKII phosphorylation sites (S383, S408 and S409) in various combinations. These included GST-β3-S408A, GST-β3-

S409A, GST-β3-S408A-S409A and GST-β3-S383A-S408A-S409A (McDonald and Moss, 1997). The wild type and mutant GST-fusion proteins were exposed to detergent-solubilised brain extracts, and purified using glutathione-agarose beads. After resolving the protein complexes by SDS-PAGE, I probed for co-purified CaMKIIα by Western blotting with a monoclonal anti-CaMKIIα antibody. A 50-kDa band was obtained in the affinity-purification input lane, and from affinity-purifications using GST-β3, GST-β3-S408A, GST-β3-S409A, GST-β3-S408A-S409A and GST-β3-S383A-S408A-S409A, but not GST alone (Fig. 3.5). These assays demonstrated that neuronal CaMKIIα was co-purified with wild type GST-β3, and that this interaction was not abolished by any of the GST-β3 serine-to-alanine mutants (GST-β3-S408A, GST-β3-S409A, GST-β3-S408A-S409A and GST-β3-S383A-S408A-S409A). This suggested that phosphorylated/dephosphorylated S383, S408 and S409 are not critical components of the interaction between native CaMKIIα-containing holoenzymes and the major intracellular domain of the $GABA_A$ receptor β3 subunit.

3.2.5 CaMKIIα-T286D Interacts Directly with the Major Intracellular Domains of $GABA_A$ Receptor β Subunits

I was next interested in identifying the site (or sites) within the β3 subunit major intracellular domain that mediates the interaction with neuronal CaMKIIα. As $GABA_A$ receptors can interact with protein kinases by direct and indirect means (Sections 1.7.2.1), and as there is currently no evidence for a specific neuronal CaMKII anchoring protein (Sections 1.8 and 3.3.1), I began by determining whether CaMKIIα binds directly to the major intracellular domain of the receptor β subunits. An important consideration in the experimental design was the previous finding that the major intracellular domains of the receptor β subunits bound selectively to the CaMKIIα mutant, CaMKIIα-T286D, which mimics T286-autophosphorylation, and not to the wild type or autophosphorylation-deficient mutant (Fig. 3.3). It was therefore essential that the kinase be phosphorylated at T286 in any further investigations of the association of CaMKIIα with the major intracellular domains of the β subunits. I therefore utilised an *in vitro* protein expression system to transcribe and translate CaMKIIα-T286D (Section 2.5.11.2) for use in an *in vitro* affinity-

A.



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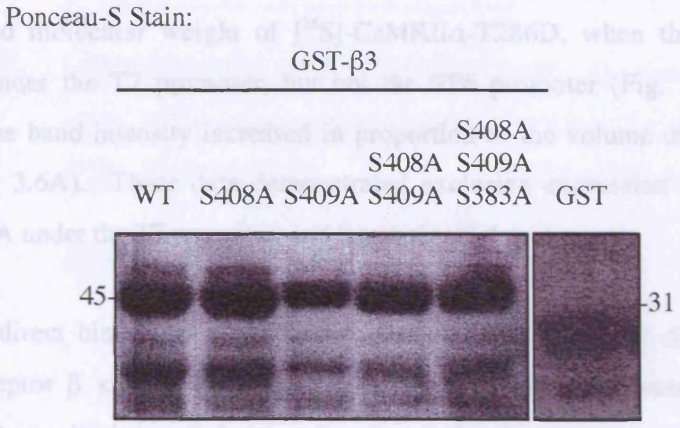


Figure 3.5. Neuronal CaMKIIα binds to the major intracellular domain of the $GABA_A$ receptor β3 subunit containing serine-to-alanine point mutations of consensus phosphorylation sites. A. Affinity-purification assays were performed using 20 μg of wild type GST-β3 (WT) and the following mutant GST-β3 fusion proteins containing serine-to-alanine point mutations: GST-β3-S408A, GST-β3-S409A, GST-β3-S408,409A and GST-β3-S408,409,383A, as well as GST alone. The GST-fusion proteins were each exposed to a detergent-solubilised brain extract, and precipitated using glutathione-agarose beads. The protein complexes were resolved by SDS-PAGE and probed for CaMKIIα by immunoblotting with a monoclonal anti-CaMKIIα antibody (CaMKIIα), and an anti-mouse alkaline phosphatase-conjugated antibody for detection. 'Input' represents 0.5% (v/v) of the brain extract used in each affinity-purification assay. **B.** Ponceau-S stain of the GST-fusion proteins in the gel in 'A.'.

purification assay. *In vitro* expression of CaMKIIα-T286D was the preferred source of kinase for several reasons. Firstly, the mutant kinase could be rapidly synthesised in a relatively inexpensive manner. Secondly, the kinase could be radiolabelled, and therefore tagged, by incorporation of [^{35}S]-labelled methionine residues during protein expression. Thirdly, direct binding assays could be performed in the absence of other 'contaminating' proteins, and finally, all of the tagged kinase proteins would be permanently modified at T286.

I first transcribed and translated CaMKIIα-T286D cDNA, *in vitro*, in the presence of [^{35}S]-labelled methionine under the control of the T7 promoter, or the SP6 promoter as a control. Following expression of the CaMKIIα-T286D cDNA, different volumes of reaction mix were resolved by SDS-PAGE, and [^{35}S]-CaMKIIα-T286D was visualised by phosphorimaging (Section 2.5.8). I detected a single band at ~51 kDa, the predicted molecular weight of [^{35}S]-CaMKIIα-T286D, when the cDNA was expressed under the T7 promoter, but not the SP6 promoter (Fig. 3.6A; Section 3.2.3.1). The band intensity increased in proportion to the volume of reaction mix loaded (Fig. 3.6A). These data demonstrated exclusive expression of CaMKIIα-T286D cDNA under the T7 promoter, and linear signal detection.

To test for direct binding of CaMKIIα to the major intracellular domains of the $GABA_A$ receptor β subunits, I used purified, bacterially expressed GST-fusion proteins of the major intracellular domain of each β subunit, and recombinant GST. The GST-fusion proteins were each exposed to [^{35}S]-CaMKIIα-T286D, and purified with glutathione-agarose beads. After the protein complexes were resolved by SDS-PAGE, [^{35}S]-CaMKIIα-T286D was visualised by phosphorimaging. I detected a single band of ~51 kDa corresponding to [^{35}S]-CaMKIIα-T286D from affinity-purification assays using GST-β1, GST-β2 and GST-β3, but not GST alone (Fig. 3.6B). This demonstrated that [^{35}S]-CaMKIIα-T286D was co-purified with GST-β1, GST-β2 and GST-β3, *in vitro*, and suggested that [^{35}S]-CaMKIIα-T286D binds directly to the major intracellular domains of the $GABA_A$ receptor β subunits, *in vitro*.

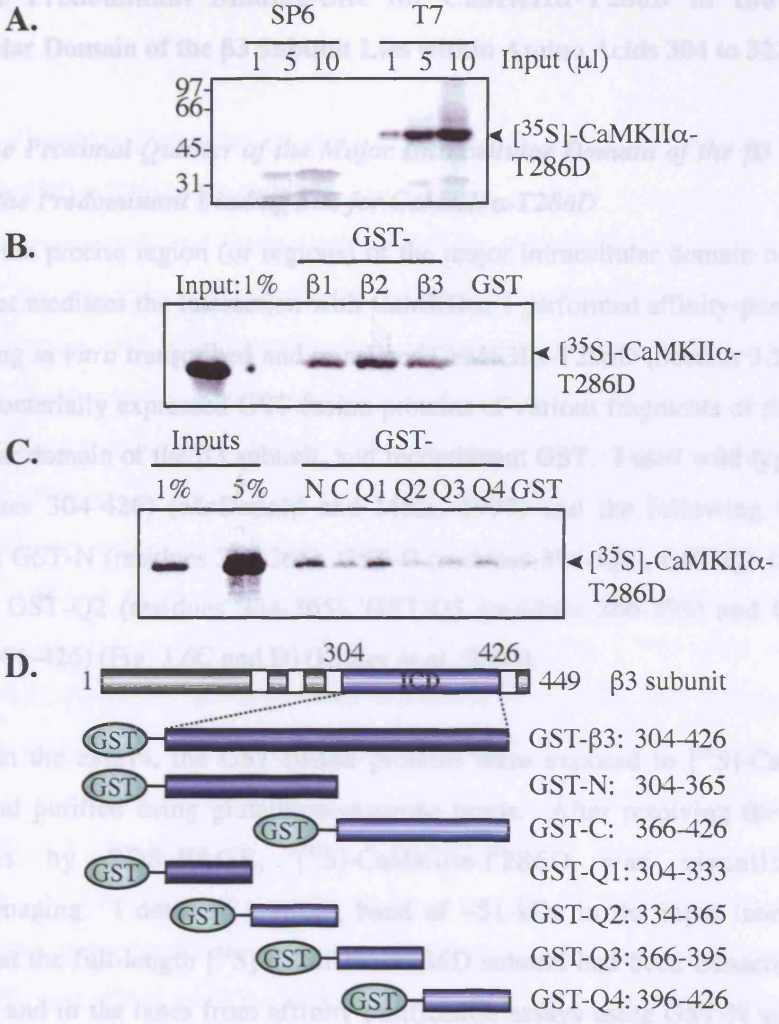


Figure 3.6. Identification of a direct docking domain for CaMKIIα–T286D in the major intracellular domain of the $GABA_A$ receptor β3 subunit. A. CaMKIIα-T286D was *in vitro* transcribed and translated in the presence of [^{35}S]-labelled methionine under the T7 promoter, or the SP6 promoter as a control. Increasing amounts of reaction mix were separated by SDS-PAGE and [^{35}S]-CaMKIIα-T286D was visualised by phosphorimaging. **B.** Affinity-purification assays were performed using GST-fusion proteins (20 µg) of the major intracellular domain (ICD) of the $GABA_A$ receptor β1, β2 and β3 subunit, or GST alone. The GST-fusion proteins were each exposed to 5 µl of reaction mix, and purified using glutathione-agarose beads. Proteins were resolved by SDS-PAGE, and [^{35}S]-CaMKIIα-T286D was visualised by phosphorimaging. 'Input' represents the given percentage (v/v) of the [^{35}S]-CaMKII-T286D mix used in each assay. **C.** Binding assays were performed as described in 'B.', using GST-fusion protein deletion constructs of the major intracellular domain of the β3 subunit (see 'D.'), or GST alone. **D.** Schematic diagram of the GST-β3 deletion constructs used in the binding assays. Those that bound strongly to CaMKIIα-T286D are coloured dark blue.

3.2.6 The Predominant Binding-Site for CaMKIIα-T286D in the Major Intracellular Domain of the β3 Subunit Lies within Amino Acids 304 to 322

3.2.6.1 The Proximal Quarter of the Major Intracellular Domain of the β3 Subunit Contains the Predominant Binding Site for CaMKIIα-T286D

To locate the precise region (or regions) of the major intracellular domain of the β3 subunit that mediates the interaction with CaMKIIα, I performed affinity-purification assays using *in vitro* transcribed and translated CaMKIIα-T286D (Section 3.2.5), and purified, bacterially expressed GST-fusion proteins of various fragments of the major intracellular domain of the β3 subunit, and recombinant GST. I used wild type GST-β3 (residues 304-426) (McDonald and Moss, 1997) and the following GST-β3 fragments: GST-N (residues 304-365), GST-C (residues 366-426), GST-Q1 (residues 304-333), GST-Q2 (residues 334-365), GST-Q3 (residues 366-395) and GST-Q4 (residues 396-426) (Fig. 3.6C and D) (Kittler *et al.*, 2005).

To perform the assays, the GST-fusion proteins were exposed to [^{35}S]-CaMKIIα-T286D, and purified using glutathione-agarose beads. After resolving the protein complexes by SDS-PAGE, [^{35}S]-CaMKIIα-T286D was visualised by phosphorimaging. I detected a strong band of ~51-kDa in the input lane, which showed that the full-length [^{35}S]-CaMKIIα-T286D subunit had been transcribed and translated, and in the lanes from affinity-purification assays using GST-N and GST-Q1 (Fig. 3.6C). I also obtained a weak ~51-kDa band from the affinity-purification assays using GST-C and GST-Q4 (although in some assays GST-C gave a stronger band), and a very weak ~51-kDa band, only just visible above background, from assays using GST-Q2 and GST-Q3 (Fig. 3.6C). I did not detect bands from binding assays using GST alone (Fig. 3.6C). These data demonstrated strong and direct binding of [^{35}S]-CaMKIIα-T286D to the N-terminal half (residues 304-365) and the first quarter (residues 304-333) of the major intracellular domain of the β3 subunit, and weak binding to the C-terminal half (residues 366-426) and the C-terminal quarters (residues 366-395; residues 396-426) of the intracellular domain (Fig. 3.6D). This suggested that [^{35}S]-CaMKIIα-T286D binds predominantly to a region within the first 30 amino acids of the major intracellular domain of the β3 subunit, and that

an additional weak CaMKII-binding site may be present in the C-terminal half of the loop.

3.2.6.2 N-Terminal Deletions of the β3 Subunit Major Intracellular Domain Disrupt CaMKIIα-T286D-Binding

To map more precisely the predominant CaMKIIα-T286D-binding site in the first quarter of the major intracellular domain of the β3 subunit, I employed *in vitro* protein expression and affinity-purification assays as described in Sections 3.2.5 and 3.2.6.1. I synthesised a second generation of GST-β3-fusion protein constructs, which contained N-terminal deletions of the major intracellular domain at intervals of ~10 amino acids (as well as a 6 amino acid C-terminal deletion) (Fig. 3.7A). This mapping strategy was used in order to preserve as much of the loop sequence and secondary-structure as possible, whilst reducing the size of the N-terminal region known to contain the [^{35}S]-CaMKIIα-T286D-binding site. The wild type and truncated GST-β3-fusion proteins were expressed in *E. coli*, and purified using glutathione-agarose beads. They included full-length GST-β3 (residues 304 to 426), and the N-terminal deletions GST-T1 (residues 313 to 420), GST-T2 (residues 323 to 420) and GST-T3 (residues 332 to 420), as well as GST alone. To perform the assays, the GST-fusion proteins were each exposed to [^{35}S]-CaMKIIα-T286D and purified using glutathione-agarose beads. Protein complexes were then resolved by SDS-PAGE, and [^{35}S]-CaMKIIα-T286D was visualised by phosphorimaging. I obtained a strong band of ~51-kDa in the input lane, and from affinity-purification assays using GST-β3 (Fig. 3.7B). A weaker band of ~51-kDa was detected from binding assays using GST-T1, GST-T2 and GST-T3 (Fig. 3.7B). I did not detect any bands with GST alone (Fig. 3.7B).

One advantage of this experimental approach was that the magnitude of [^{35}S]-CaMKIIα-T286D-binding to each GST-fusion protein could be determined by measuring the intensity of each [^{35}S]-CaMKIIα-T286D band using Bio-Rad Quantity One software. This type of analysis enabled us to quantitatively examine the extent to which each successive N-terminal deletion was able to perturb binding to the kinase. To perform the analysis, I first ensured that the GST-fusion proteins were

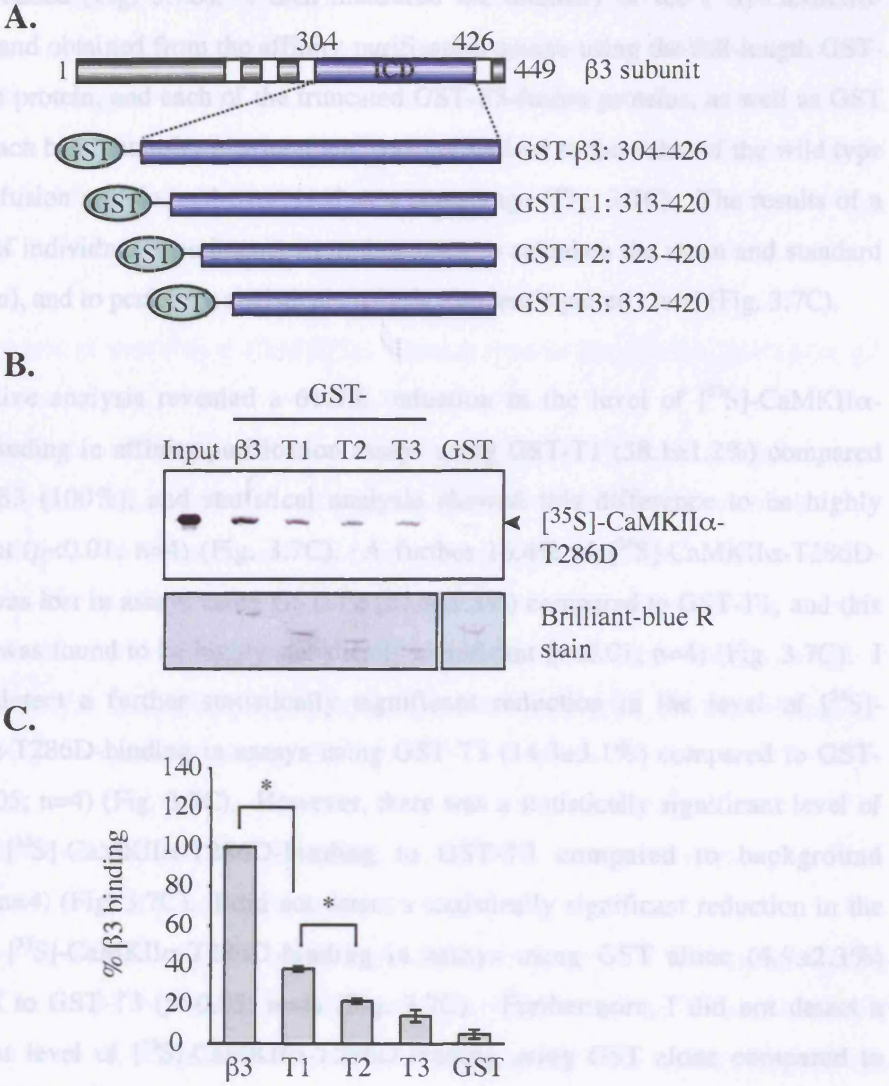


Figure 3.7. N-terminal deletions of the major intracellular domain of the β3 subunit disrupt the binding of CaMKIIα-T286D. A. Schematic diagram of the GST-β3 deletion constructs used in this binding study. ICD: major intracellular domain. **B. Upper panel:** affinity-purification assays were performed using 20 μg of the GST-fusion proteins described in 'A.', as well as GST alone. The GST-fusion proteins were each exposed to [^{35}S]-CaMKIIα-T286D, and precipitated using glutathione-agarose beads. Proteins were then resolved by SDS-PAGE, and [^{35}S]-CaMKIIα-T286D was visualised by phosphorimaging. 'Input' represents 10% (v/v) of the assay mix used in each of the binding assays. **Lower panel:** brilliant-blue R stain of the gel in the upper panel showing equal loading of the GST-fusion proteins. **C.** Quantitation of 'B.'. The band intensity of each truncated GST-fusion protein was normalised to full-length GST-β3 and expressed as a percentage. The results of several individual experiments were then compared using the Student's paired *t* test. * denotes $p<0.01$ (mean ± sem; n = 4).

equally loaded (Fig. 3.7B). I then measured the intensity of the [^{35}S]-CaMKIIα-T286D band obtained from the affinity-purification assays using the full-length GST-β3-fusion protein, and each of the truncated GST-β3-fusion proteins, as well as GST alone. Each band intensity measurement was normalised to the value of the wild type GST-β3-fusion protein, and expressed as a percentage (Fig. 3.7C). The results of a number of individual experiments were then used to calculate the mean and standard error (sem), and to perform a statistical analysis (Student's paired *t* test) (Fig. 3.7C).

Quantitative analysis revealed a 61.9% reduction in the level of [^{35}S]-CaMKIIα-T286D-binding in affinity-purification assays using GST-T1 (38.1±1.2%) compared to GST-β3 (100%), and statistical analysis showed this difference to be highly significant ($p<0.01$; n=4) (Fig. 3.7C). A further 16.4% of [^{35}S]-CaMKIIα-T286D-binding was lost in assays using GST-T2 (21.8±1.3%) compared to GST-T1, and this decrease was found to be highly statistically significant ($p<0.01$; n=4) (Fig. 3.7C). I did not detect a further statistically significant reduction in the level of [^{35}S]-CaMKIIα-T286D-binding in assays using GST-T3 (14.3±3.1%) compared to GST-T2 ($p>0.05$; n=4) (Fig. 3.7C). However, there was a statistically significant level of residual [^{35}S]-CaMKIIα-T286D-binding to GST-T3 compared to background ($p<0.05$; n=4) (Fig. 3.7C). I did not detect a statistically significant reduction in the level of [^{35}S]-CaMKIIα-T286D-binding in assays using GST alone (4.9±2.3%) compared to GST-T3 ($p>0.05$; n=4) (Fig. 3.7C). Furthermore, I did not detect a significant level of [^{35}S]-CaMKIIα-T286D-binding using GST alone compared to background ($p>0.05$; n=4) (Fig. 3.7C).

These data collectively demonstrated that the first nine residues (304 to 312), and possibly the last six residues (421 to 426), of the major intracellular loop of the β3 subunit played a major role in binding [^{35}S]-CaMKIIα-T286D. The results also showed that residues 313 to 322 mediated some binding to [^{35}S]-CaMKIIα-T286D. Residues 323 to 331 did not appear to be involved in binding [^{35}S]-CaMKIIα-T286D, at least in the absence of the N-terminal loop region (residues 304-322). Given that [^{35}S]-CaMKIIα-T286D binds GST-Q1 (304-333) (Fig. 3.6C and D), these results suggested that the first 19 amino acids of the major intracellular domain of the β3

subunit are sufficient to mediate an interaction with [^{35}S]-CaMKIIα-T286D, *in vitro*. They also suggest that this association mediates the majority (~80%) of the total binding of the kinase to GST-β3. Together with the results from Section 3.2.6.1, these data further suggested that a secondary binding site is present between residues 332 and 420, and that this region binds a much smaller proportion (~20%) of [^{35}S]-CaMKIIα-T286D than the N-terminal site, *in vitro*.

3.2.7 Analysis of the Major CaMKIIα-Binding Site in the $GABA_A$ Receptor β3 Subunit

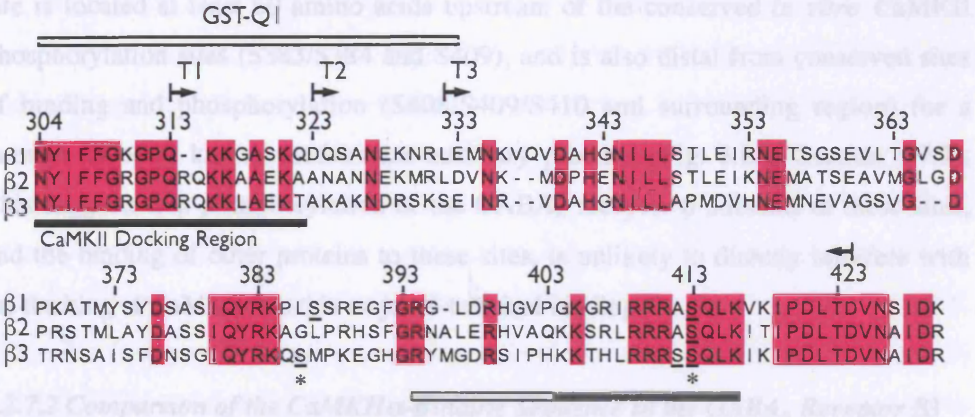
I have shown that native CaMKII binds non-selectively to various $GABA_A$ receptor subunits (Section 3.2.1), and that recombinant CaMKIIα-T286D binds to some or all of the first 19 N-terminal amino acids of the major intracellular domain of the receptor β3 subunit (Section 3.2.6). I was therefore interested in examining whether this CaMKIIα-T286D-binding region is conserved between the β subunit isoforms, and in comparing the docking sequence to other known CaMKII-binding domains to try to identify a common binding motif.

3.2.7.1 Comparison of the CaMKIIα-Binding Sequence in the $GABA_A$ Receptor β3 Subunit with the other β Subunit Sequences

I first analysed the sequence similarity of the major CaMKII-docking region identified in the β3 subunit with that of the homologous regions in the β1 and β2 subunits because recombinant CaMKIIα has been shown to bind directly to all the $GABA_A$ receptor β subunits (Section 3.2.5; Fig. 3.6B). I annotated murine sequences of the major intracellular domains of $GABA_A$ receptor β subunits (Fig. 3.8A), as this is the species of β subunit used in all binding assays in this study. Multiple sequence alignments were constructed using ClustalW (Higgins, 1994), and manual editing using the Jalview Java Alignment Editor (Clamp *et al.*, 2004).

I detected a high degree of amino acid identity within the major CaMKII-docking region comprising residues 304 to 322 (Fig. 3.8A). Interestingly, the following nine residues, residues 323-331, which did not appear to mediate any binding to CaMKII, displayed little sequence identity (Fig. 3.8A). It is likely therefore that the major

A.



B.

Protein	Sequence of CaMKII Docking Region
β3 (304-322)	NYIFFGRGPQRQKKLAEKT
CaMKIIN (43-68)	RPPKLCQICRAKRVVIEDDRIDDVLK
CaMKII (281-302)	MHRQETVDCLKKFNARRKLKGA
NR2B (1290-1309)	AQKKNRNKLRRQHSYDTFVDL
NR2A (1413-1419)	SRDSRGH
NR1a (845-861)	RKQMQLAFAAVNVWRKN
DENSIN-180 (1353-1380)	PYEGNINKVTIQQFQSPLPIQIPSSQAT
synGAPβ (1146-1166)	SPSLQADAGGGGAAPGGPRHG
Eag K^+ channel (773-794)	PKLQASQATLARQDTTDEGGEV

(Adapted from Colbran, 2004 and Merrill et al., 2005)

Figure 3.8. Sequence analysis of the proposed CaMKIIα–docking region in the $GABA_A$ receptor β3 subunit. A. Sequence alignment of the murine β subunit major intracellular domains. Black letters on pink boxes indicate 100% amino acid identity between proteins. Numerals indicate amino acid position of the given alignment, beginning with respect to the β3 subunit. The β3 subunit truncations, T1 (313-420), T2 (323-420) and T3 (332-420) are indicated with arrows. The open bar indicates the GST-Q1 binding-partner. The black bar indicates the proposed CaMKII docking domain in the β3 subunit, the dark grey bar indicates the PKC binding region in the β3 subunit and the light-grey bar indicates the RACK-1 binding region in the β3 subunit. * indicates the position of the conserved *in vitro* CaMKII phosphorylation sites, and major known sites of phosphorylation are underlined. **B.** Sequence comparison of some known CaMKII-docking regions less than 30 amino acids in length. The regions underlined indicate the only sequence similarity to be found, which occurs between a region of NR2B (residues 1290 to 1309) and the CaMKII autoinhibitory subdomain (residues 281 to 302). A single consensus binding motif is not apparant.

CaMKII-docking region is conserved between the β subunit isoforms. This binding site is located at least 60 amino acids upstream of the conserved *in vitro* CaMKII phosphorylation sites (S383/S384 and S409), and is also distal from conserved sites of binding and phosphorylation (S408/S409/S410 and surrounding region) for a number of other kinases and kinase-auxiliary proteins (Fig. 3.8A; Section 1.7.2). This suggests that phosphorylation of the $GABA_A$ receptor β subunits at these sites, and the binding of other proteins to these sites, is unlikely to directly interfere with the docking of CaMKIIα to this major N-terminal binding site.

3.2.7.2 Comparison of the CaMKIIα-Binding Sequence in the $GABA_A$ Receptor β3 Subunit with CaMKIIα-Binding Sequences in other Proteins

I was also interested in comparing the CaMKII-binding region identified in the $GABA_A$ receptor β3 subunit with CaMKII-docking sites that have been described for other proteins to determine whether there is any sequence homology. Figure 3.8B shows the amino acid sequences of some known CaMKII-binding regions in other proteins that are less than 30 amino acids in length. Apart from a small degree of homology between a region of NR2B (residues 1290-1309) and the autoinhibitory domain of CaMKII (residues 281-302), I did not detect any significant sequence homology between the protein regions (Fig. 3.8B). This suggested that CaMKII does not interact with its binding-partners via a single consensus motif.

3.3 Discussion

The differential targeting of protein kinases to $GABA_A$ receptor scaffolds has been shown to facilitate phosphorylation and to regulate receptor activity (Section 1.7; Moss and Smart, 2001; Kittler and Moss, 2003). It is therefore fundamental to our understanding of inhibitory neurotransmission, to understand the mechanisms by which protein kinases are recruited to $GABA_A$ receptors. CaMKII has been implicated in regulating the activity of neuronal $GABA_A$ receptors, and has been shown to phosphorylate some receptor subunits, *in vitro* (Section 3.1; McDonald and Moss, 1994, 1997). However, CaMKII remains to be identified as a $GABA_A$ receptor-associated kinase.

In this chapter, I investigated the physical relationship between the $GABA_A$ receptor and CaMKII, with the aim of understanding how CaMKII is targeted to receptor complexes to mediate phosphorylation. This study has led to the identification of a stable, phosphorylation-dependent physical interaction between CaMKII and the $GABA_A$ receptor. I demonstrated that this association occurs in brain, and that the native kinase binds to the major intracellular domain of various receptor subunits without any apparent selectivity. In addition, I found that the major intracellular domains of the receptor β subunits bound selectively to a recombinant form of CaMKIIα that contains a threonine-to-aspartate point mutation at T286, which mimics T286-autophosphorylation and induces constitutive activity. Although the interaction between CaMKIIα and the major intracellular domain of the β3 subunit was shown to require kinase phosphorylation, it appeared to be independent of $GABA_A$ receptor subunit phosphorylation. This finding was supported by subsequent mapping studies, which identified a direct CaMKIIα-docking site at the N-terminal end of the major intracellular domain of the β3 subunit. These studies further revealed that this binding site is located within the first 19 amino acids of the major intracellular domain, at least 60 residues upstream of the consensus CaMKII *in vivo* phosphorylation motifs. I also demonstrated that this binding region displays a large degree of sequence homology across all the β subunit isoforms. I propose that this phosphorylation-dependent interaction underlies the differential targeting of CaMKIIα to $GABA_A$ receptors, and that this may facilitate the regulation of $GABA_A$ receptor functionality in brain.

3.3.1 The Nature of CaMKIIα-$GABA_A$ Receptor Complexes

My studies demonstrated that CaMKIIα-containing holoenzymes are able to form native complexes with $GABA_A$ receptors in brain. I also showed that native CaMKIIα binds directly and/or indirectly to the major intracellular domain of various $GABA_A$ receptor subunits, including α1, β1-3, γ1-3 and δ. Most neuronal $GABA_A$ receptors are thought to comprise α, β and γ subunits (Section 1.3; Fig. 1.1). A δ subunit is also believed to replace the γ subunit in some receptor assemblies (Section 1.3; Fig. 1.1). It is therefore possible that CaMKIIα-containing holoenzymes form native complexes with most $GABA_A$ receptor populations in brain. As CaMKII

holoenzymes can be assembled as subunit heteromers as well as homomers, it is also possible that other neuronal CaMKII isozymes are also part of the native CaMKIIα-$GABA_A$ receptor complex (Section 1.8; Fig. 1.2). Furthermore, as the ratio of substituent isozymes in a given holoenzyme influences properties of CaMKII such as calmodulin sensitivity and protein-protein interactions (Section 1.8; see below), then the presence of other isozymes could have a significant impact on any CaMKII-dependent modulation of $GABA_A$ receptors, which may itself be characterised by receptor subtype.

The large macromolecular structure of CaMKII provides numerous spatially dispersed points of contact for the binding of $GABA_A$ receptor subunits. Since the majority of neuronal $GABA_A$ receptor assemblies are thought to comprise 2α, 2β and 1γ or 1δ subunits (Section 1.3; Fig. 1.1), then it is likely that most receptor complexes contain at least three subunit binding-partners for CaMKIIα. This complexity may be further increased by the presence of multiple CaMKII binding sites in the major intracellular domains of the receptor subunits (discussed below). Furthermore, although my findings demonstrated that this region is sufficient to mediate an interaction with native CaMKIIα, additional points of contact may also exist between CaMKII and other subunit domains, such as the minor intracellular loop between TM1 and TM2, and the TM regions (Fig. 1.1). Notably, direct binding-partners have yet to be identified for these regions. However, TM4 has been shown to facilitate the recruitment of gephyrin to clusters of postsynaptic $GABA_A$ receptors (Alldred *et al.*, 2005).

In this study, I found that the interaction between recombinant CaMKIIα and each of the $GABA_A$ receptor β subunits is dependent upon T286 phosphorylation. This is similar to the enhanced binding of PKC to $GABA_A$ receptor β subunits following its activation and phosphorylation by phorbol esters or BDNF (Brandon *et al.*, 1999; Jovanovic *et al.*, 2004). A key question, however, is how phosphorylation of CaMKII regulates binding. One possibility is that the phosphorylated T286 residue and a flanking region comprise the binding site for the receptor subunits. The autoregulatory domain (which contains this region) (Fig. 1.2) has been shown to

mediate binding to the neuronal protein Camguk in *Drosophila*, although this has not yet been shown with the mammalian homologue, CASK (Lu *et al.*, 2003). Alternatively, the conformational change in isozyme structure induced by phosphorylation of T286 (Section 1.8) may expose a receptor-binding site elsewhere in the protein. Other CaMKII subunit domains have been shown to mediate binding of the kinase to postsynaptic proteins. For example, the catalytic domain has been shown to mediate binding to α-actinin (Walikonis *et al.*, 2001; Dhavan *et al.*, 2002), the NR2B (Bayer *et al.*, 2001; Strack *et al.*, 2000) and NR2A (Gardoni *et al.*, 1999, 2001) subunits of NMDA-type glutamate receptors, the cyclin-dependent kinase 5 activators, p35 and p39, (Dhavan *et al.*, 2002) and the Eag K^+ channel (Sun *et al.*, 2004). Furthermore, the association domain has been shown to mediate binding to densin-180 (Strack *et al.*, 2000; Walikonis *et al.*, 2001) and MUPP-1 (Krapivinsky *et al.*, 2004). Interestingly, intracellular application of pre-activated CaMKII subunits containing the catalytic and autoregulatory domains, but not the association domain, to cerebellar granule cells has been shown to increase the amplitude of both GABA-mediated currents and spontaneous IPSCs (C.M. Houston, unpublished data). Addition of pre-activated CaMKII (catalytic and autoregulatory domains only) to NG108-15 cells expressing $GABA_A$ receptor subunits also induces potentiation of GABA-mediated currents (C.M. Houston, unpublished data). This suggests that any interaction between the association domain of CaMKII and the receptor subunits is not necessary for functional modulation of $GABA_A$ receptors. It is perhaps important to note that a binding sequence for the $GABA_A$ receptor β subunits may also lie within an isozyme-specific domain of CaMKII (Section 1.8; Fig. 1.2). If this is the case, then the interaction between CaMKII and the $GABA_A$ receptor may also be regulated in an isozyme-specific manner. Indeed, such a binding mechanism has been shown to determine the interaction between CaMKIIβ and F-actin (Shen *et al.*, 1998), and CaMKIIα and densin-180 (Walikonis *et al.*, 2001). Additional experiments that determine the region(s) of CaMKII involved in binding $GABA_A$ receptor subunits will therefore be of interest.

Although my findings indicate that phosphorylation of CaMKII at T286 induces binding of the kinase to the $GABA_A$ receptor β subunits, my data does not necessarily

suggest that this modification alone is sufficient for binding. My studies utilised a recombinant form of CaMKIIα containing a permanent T286D point mutation to mimic autophosphorylation and induce constitutive activity. However, autophosphorylation at T305, T306 and S314 (and possibly other sites that have yet to be characterised) is known to follow autophosphorylation at T286 and the subsequent removal of calcium/CaM (Hanson and Schulman, 1992; Hashimoto *et al.*, 1987; Patton *et al.*, 1990; LeVine *et al.*, 1985; Miller *et al.*, 1988; Lengyel *et al.*, 2000). Furthermore, the phosphorylation state of T305/T306 is known to be involved in regulating the localisation of CaMKII at the PSD of excitatory synapses (Shen *et al.*, 2000; Elgersma *et al.*, 2002; Weeber *et al.*, 2003). It is therefore possible that autophosphorylation of one or all of these residues (T305, T306 and S314) also plays a role in regulating the association of CaMKII with $GABA_A$ receptor complexes. Indeed, additional phosphorylation of CaMKIIα-T286D may contribute to the altered migration of this kinase mutant during SDS-PAGE (to ~51 kDa) compared to CaMKIIα-WT and CaMKIIα-T286A, which both migrate to 50 kDa (Section 3.2.3). It is also possible that the upward band shift is caused by the substitution of the neutral threonine residue for an aspartate residue.

My studies further revealed that EGTA is required for the binding of constitutively active CaMKIIα to the $GABA_A$ receptor β3 subunit, *in vitro*. In the binding studies, kinase autophosphorylation at T286 was independent of calcium/CaM (due to the use of a T286D point mutation to mimic autophosphorylation) and, hence, EGTA-mediated chelation of calcium. However, the chelation of free calcium and zinc ions by EGTA might promote (or disinhibit) the interaction between CaMKIIα-T286D and the receptor β3 subunit by stabilising/destabilising the conformation of the kinase and/or receptor proteins, or by regulating the phosphorylation state of other CaMKII sites. For example, the chelation of calcium by EGTA may be necessary to ensure phosphorylation of T305, T306 and S314 (see above). Furthermore, phosphorylation of CaMKII may be altered by the chelation of zinc, as this ion has been shown to have an effect on the phosphorylation state and activity of this kinase (Lengyel *et al.*, 2000).

My studies revealed that recombinant CaMKIIα interacts directly with the major intracellular domain of each $GABA_A$ receptor β subunit. A major binding site for recombinant CaMKIIα was also found to include some or all of the first 19 amino acids of the major intracellular domain of β3 subunit. The high sequence homology between the β subunit isoforms in this region further suggested that the CaMKII binding site is conserved within the β subunit family. However, in the absence of a known consensus binding-motif for CaMKII, the precise CaMKII-binding sequence in this region of the $GABA_A$ receptor β subunits remains to be delineated. The lack of sequence homology between known CaMKII-docking sites of various neuronal proteins implies that CaMKII does not interact with all its binding-partners via a single mechanism. It is possible that some CaMKII-binding motifs consist of general 'basic-rich' or 'charged' regions. CaMKII binding may also be determined by three-dimensional motifs in the protein structure.

My findings suggested that CaMKII interacts with at least two distinct sites within the major intracellular domain of the $GABA_A$ receptor β3 subunit. A major CaMKIIα-docking site was located at the N-terminal end of the major intracellular domain, and a secondary weak binding site appeared to be present in the C-terminal half of the loop. Multiple CaMKII-binding sites have been identified in the NMDA receptor NR2B subunit (Leonard *et al.*, 1999; Strack and Colbran, 1998; Strack *et al.*, 2000; Bayer *et al.*, 2001), although not all CaMKII-binding partners are thought to form multivalent interactions with the kinase (Merrill *et al.*, 2005). Indeed, the weak binding observed in the C-half of the loop may be due to a non-specific low-affinity association of the kinase around the *in vitro* substrate sites, S383 and S409. Biochemical and electrophysiological experiments using a synthetic peptide of the N-terminal CaMKII-binding sequence may help determine the importance of this site in mediating binding of CaMKII to $GABA_A$ receptor complexes, and the role of CaMKII-binding in CaMKII-dependent phosphorylation and functional modulation of $GABA_A$ receptors.

The major CaMKII binding site in the receptor β3 subunit is located at least 60 amino acids upstream of the *in vitro* CaMKII phosphorylation sites, S383 and S409. This

apparently distinct location may enhance the specificity of β subunit phosphorylation by CaMKII (Johnson and Hunter, 2005), and is consistent with my finding that S383, S408 and S409 were not critical components of the interaction between CaMKIIα and the β3 subunit. It is possible that S383, S408 and S409 are components of the secondary binding site, and that phosphorylation of these residues regulates (inhibits or enhances) the binding of CaMKII. This could be explored by performing binding assays with receptor β3 subunits that have been phosphorylated or mock phosphorylated by purified CaMKII, or that contain serine-to-aspartate point mutations that mimic phosphorylation. Notably, binding of PKC to the receptor β subunits is negatively regulated by phosphorylation at the PKC sites (Brandon *et al.*, 1999, 2002; Jovanovic *et al.*, 2004) (Section 1.7.2.1.1).

Although the interactions between protein kinases and $GABA_A$ receptors can be direct, previous binding studies have shown that they often involve auxiliary proteins (Section 1.7.2.1). Both PKC and PKA engage in co-operative binding; PKA is localised to receptors by the AKAP-79/150 anchoring protein, and the RACK-1 auxiliary protein enhances the activity of receptor-associated PKC (Sections 1.7.2.1.1 and 1.7.2.1.2). Whilst my findings suggest that CaMKIIα-containing holoenzymes bind directly to $GABA_A$ receptors, I cannot exclude the possibility that CaMKIIα forms an indirect association with receptor complexes via an adaptor protein. As CaMKIIα interacts with various receptor subunits, it is possible that such a linker protein is a common subunit binding-partner. Alternatively, there may be several distinct adaptor proteins. Although adaptor proteins are not necessary for formation of CaMKII-$GABA_A$ receptor complexes *in vitro*, they may be required to localise the kinase to the receptor in intact cells. These proteins might be bound to receptor subunits, or be detached from receptor scaffolds but in close enough molecular proximity to promote the formation of kinase-receptor complexes. Furthermore, such auxiliary proteins may be static elements of receptor scaffolds or the immediate surroundings, or be subject to dynamic localisation.

Although inhibitory synaptic scaffolds comprise a diverse collection of signalling and scaffolding proteins (Sections 1.4, 1.5, 1.6 and 1.7), there are currently few candidate

CaMKII-anchoring proteins present. Both PP1 and PP2A are involved in dephosphorylating CaMKII, and have been localised to $GABA_A$ receptors (Section 1.7.2.2), but it is not yet known whether these phosphatases are involved in recruiting CaMKII to inhibitory synapses. It is possible that non-kinase components of the CaMKII holoenzyme function to target kinase activity to $GABA_A$ receptors. In skeletal muscle, the incorporation of an αKAP protein (a CaMKIIα subunit in which the kinase domain is substituted with a hydrophobic region) into a CaMKII holoenzyme has been shown to induce membrane targeting and altered regulation of ryanodine receptor activity (Bayer *et al.*, 1998; Section 1.8). However, the presence of αKAP proteins in neurons as integral CaMKII anchoring units has not yet been investigated, and so any function αKAPs may have in directing CaMKII to cell-surface $GABA_A$ receptors remains highly speculative.

3.3.2 Functional Implications of Native CaMKIIα-$GABA_A$ Receptor Complexes

In this study, I have provided the first biochemical evidence that CaMKII forms a native complex with $GABA_A$ receptors in brain. This is consistent with a previous ultrastructural study of CaMKII localisation in the basolateral amygdala demonstrating CaMKII immunoreactivity at the PSD of some GABAergic synapses (McDonald *et al.*, 2002). However, similar electron microscopy studies of the sub-cellular localisation of CaMKII in the thalamus, neocortex (Liu and Jones, 1996) and CA1 region of the hippocampus (Liu and Jones, 1997) do not detect CaMKIIα in the PSD of symmetrical synapses. Furthermore, double-labelling immunofluorescence studies do not detect colocalisation of CaMKII and GABA in the majority of forebrain regions (Benson *et al*, 1992; McDonald *et al*, 2002).

My finding that the interaction of CaMKIIα with $GABA_A$ receptor subunits is dependent upon phosphorylation at T286 supports the idea that CaMKIIα is translocated to, and binds $GABA_A$ receptors at inhibitory synaptic sites following autophosphorylation. Indeed, CaMKII has been shown to undergo activity-dependent translocation to the PSD of excitatory synapses (Strack *et al.*, 1997b; Shen and Meyer, 1999; Shen *et al.*, 2000; Gleason *et al.*, 2003; Otmakhov *et al.*, 2004; Dosemeci *et al.*, 2001). Although phosphorylation of T286 is not believed to be

essential for such translocation (Shen and Meyer, 1999; Shen *et al.*, 2000), it is thought to be involved in the retention of the kinase at these postsynaptic sites (Shen *et al.*, 2000). Furthermore, phosphorylation of T286 has been shown to either enhance or be required for the interaction of CaMKII with a number of postsynaptic proteins, including the NR1-, NR2A- and NR2B-type NMDA receptor subunits (Bayer *et al.*, 2001; Strack and Colbran, 1998; Gardoni *et al.*, 1999) and densin-180 (Strack *et al.*, 2000, Walikonis *et al.*, 2001). Some of these molecular partnerships exist to recruit CaMKII to specific sub-cellular locations (Bayer *et al.*, 2001; Shen *et al.*, 1998; Strack *et al.*, 2000), whereas others are involved in 'trapping' active/inactive forms of CaMKII at, or close to, substrate sites (Bayer *et al.*, 2001; Sun *et al.*, 2004; Lu *et al.*, 2003). It is possible that the interaction between CaMKII and $GABA_A$ receptors fulfils either or both of these roles.

Activity-dependent translocation of CaMKII may explain some of the inconsistencies in the published literature regarding the sub-cellular distribution of CaMKII in neurons. For example, the localisation studies described above were performed using neurons that were fixed under basal conditions. The variation in CaMKII localisation observed in these studies may therefore be due to neuron-specific differences in the activity and autophosphorylation of CaMKII in the basal state.

The putative translocation of CaMKIIα to inhibitory synaptic scaffolds might occur over small or large distances and be either transient or long lasting. It is possible that in the inactivated state, CaMKIIα is associated with synaptic scaffolds near to, but physically distinct from $GABA_A$ receptors, and that autophosphorylation at T286 induces binding to receptor subunits. However, the apparent low occupancy of CaMKII at the PSDs of symmetrical synapses under basal conditions (McDonald *et al.*, 2002) suggests that soluble CaMKII is shuttled to $GABA_A$ receptors following activation and autophosphorylation from a location beyond the active zone of the synapse. If this is the case, then the activity-dependent targeting of CaMKII to $GABA_A$ receptors would be expected to increase the proportion of GABAergic synapses containing CaMKII, and hence detection of the kinase. There are several potential advantages of activity-dependent targeting of CaMKII to inhibitory

synapses. For example, such translocation would provide an additional level of regulation in the neuronal signalling network, which would enhance the specificity of intracellular signalling events. Furthermore, this mechanism of spatial regulation could enable fine control of the concentration of activated CaMKII at the synapse, which could be important if the kinase is susceptible to rapid and prolonged kinase deactivation by the local action of protein phosphatases or degradative mechanisms.

My finding that CaMKIIα interacts with $GABA_A$ receptors is consistent with previous reports showing phosphorylation of the major intracellular domains of the $GABA_A$ receptor subunits by purified CaMKII *in vitro* (McDonald and Moss, 1994, 1997), and functional modulation of $GABA_A$ receptors following intracellular application of CaMKII to heterologous cells and neurons (Section 3.1). Nevertheless, the binding of CaMKIIα to $GABA_A$ receptors does not necessarily indicate that phosphorylation will occur, as protein kinases have been shown to interact with non-substrate proteins (Johnson and Hunter, 2005). Indeed, given the large size of the multimeric CaMKII holoenzyme and evidence suggesting that CaMKII can self-associate in neurons to form large clusters (Hudmon *et al.*, 2005), it is possible that CaMKII functions as a structural hub of inhibitory synapses, acting as a scaffold for various synapse-associated proteins. However, as my results suggested that phosphorylation of CaMKIIα at T286 is required for the association with $GABA_A$ receptor subunits, it is likely that the bound kinase is enzymatically active. Furthermore, as $GABA_A$ receptor subunits are substrates of CaMKII *in vitro* (McDonald and Moss, 1994, 1997), then it is likely that subunit phosphorylation is a consequence of binding. Modification of the phosphorylation state of $GABA_A$ receptors is believed to play a pivotal role in regulating channel activity and the efficacy of inhibitory neurotransmission (Section 1.7). Phosphorylation of $GABA_A$ receptors by CaMKII may therefore play a key role in modulating the strength of GABAergic synapses. A more detailed discussion of the functional implications of CaMKII-dependent regulation of $GABA_A$ receptors can be found in Chapters 4 and 6.

In addition to being a component of post-synaptic $GABA_A$ receptor scaffolds, CaMKII may also bind presynaptic and extrasynaptic $GABA_A$ receptors. For

example, CaMKII may interact with presynaptic $GABA_A$ receptors localised in glutamatergic axon terminals (Engelman and MacDermott, 2004), where CaMKII-mediated phosphorylation of synapsin I is involved in neurotransmitter release (Lin *et al.*, 1990; Benfenati *et al*, 1992; Waxham *et al*, 1993). As well as being associated with $GABA_A$ receptors on the cell-surface of neurons, CaMKII may also bind sub-membranous and intracellular $GABA_A$ receptors, such as those in endocytic or recycling pathways. These interactions might take place in axonal, somatic or dendritic compartments, and have a significant effect on the trafficking of $GABA_A$ receptors to and from inhibitory synapses. Further investigations of the sub-cellular location of $GABA_A$ receptor-CaMKIIα complexes will be critical in unravelling the potentially diverse functional effects of such interactions in neurons.

3.3.3 Potential Dynamics of $GABA_A$ Receptor Scaffolds

I have identified CaMKII as a novel $GABA_A$ receptor-associated kinase. CaMKII therefore joins a growing family of signalling proteins that interact with $GABA_A$ receptors. Several members of this family include enzymes that modify the phosphorylation state of $GABA_A$ receptors, and auxiliary proteins that support protein kinase or phosphatase activity (Fig. 3.9; Section 1.7). Although having multiple protein kinase and phosphatase effectors may enhance the specificity of signal transduction to $GABA_A$ receptors, the dynamic interplay of these signalling proteins at individual receptor subunits and complexes remains poorly understood. Whereas PKC is recruited to a distinct region of the β subunit major intracellular domain (Section 1.7.2.1.1) to CaMKII, the binding domains of other protein kinases and phosphatases remain unknown. Binding of CaMKII may therefore compete with or be facilitated by these enzymes or other $GABA_A$ receptor-associated proteins.

Interestingly, CaMKII may compete with the ubiquitin-like protein Plic-1 for binding to $GABA_A$ receptor α and β subunits. Plic-1 has been shown to bind the first ten amino acids (NYFTKRGYAW) of the major intracellular domain of the receptor α1 subunit (Bedford *et al.*, 2001). Sequence analysis has further revealed that the first half of this sequence (NYFTK) is conserved in the receptor α1-3, α6 and β subunits (Bedford *et al.*, 2001). I have demonstrated that CaMKII binds to the receptor β

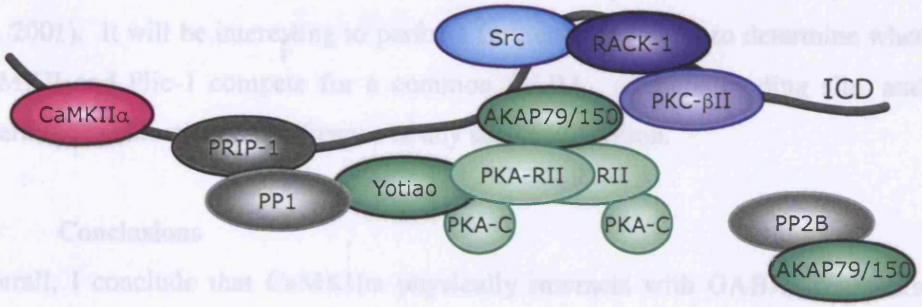


Figure 3.9. CaMKII is a novel $GABA_A$ receptor-associated kinase. Schematic diagram of protein kinase and phosphatase signalling complexes at the major intracellular domain (ICD) of the $GABA_A$ receptor β3 subunit. Known interactions are shown. The schematic does not illustrate exact points of contact.

subunits at a site located within the first 19 amino acids of the major intracellular domain. It is therefore possible that CaMKII and Plic-1 bind to a common region in these subunits. If so, then this would suggest that the interaction identified in this study between CaMKII and the α1 subunit is direct. Furthermore, it would suggest that CaMKII and Plic-1 compete for the α1 and β binding regions. Plic-1 has been localised to GABAergic synapses as well as various intracellular compartments (Bedford *et al.*, 2001). However, the predominant effect of Plic-1 on $GABA_A$ receptor function appears to involve the stabilisation of intracellular pools of $GABA_A$ receptors to facilitate the insertion of receptors into the plasma membrane (Bedford *et al.*, 2001). It will be interesting to perform further experiments to determine whether CaMKII and Plic-1 compete for a common $GABA_A$ receptor binding site, and to determine the functional significance of any such competition.

3.4 Conclusions

Overall, I conclude that CaMKIIα physically interacts with $GABA_A$ receptors in brain. CaMKII binds non-selectively to the major intracellular domains of various receptor subunits. The interaction with the receptor β subunits is dependent upon phosphorylation of the kinase at T286, but does not appear to require $GABA_A$ receptor subunit phosphorylation. Indeed, a major binding site for CaMKII has been identified within the proximal region of the major intracellular domain of the β subunits, at least 60 amino acids upstream of the *in vitro* CaMKII phosphorylation sites. The stable, phosphorylation-dependent interaction of CaMKIIα with $GABA_A$ receptors may underlie the ability to differentially target CaMKII to $GABA_A$ receptor complexes. This may facilitate the regulation of receptor activity in brain, and therefore have significant consequences for the efficacy of inhibitory neurotransmission and plasticity at GABAergic synapses.

CHAPTER 4

CaMKII-Dependent Phosphorylation of $GABA_A$ Receptors

4.1 Background

In Chapter 3, I demonstrated that CaMKIIα interacted with $GABA_A$ receptors in brain, and that the kinase bound non-selectively to the major intracellular domains of various $GABA_A$ receptor subunits. This interaction did not appear to require $GABA_A$ receptor subunit phosphorylation, but was dependent upon phosphorylation of CaMKII at T286. Although I established a physical interaction between neuronal CaMKII and $GABA_A$ receptors, such an association will not necessarily coincide with phosphorylation (Johnson and Hunter, 2005). I was therefore interested in determining whether $GABA_A$ receptors are substrates of CaMKII in neurons.

Several lines of evidence suggest that CaMKII may be involved in phosphorylating $GABA_A$ receptors in neurons. Firstly, the results of electrophysiological studies based on the intracellular application of CaMKII and modulation of postsynaptic calcium/CaM signalling cascades suggest that CaMKII is involved in modulating GABAergic neurotransmission (see Section 3.1 for a detailed discussion; Kano *et al.*, 1996; Kawaguchi and Hirano, 2002; Wei *et al.*, 2004; Wang *et al.*, 1995; Aguayo *et al.*, 1998). Secondly, biochemical studies have shown that CaMKII phosphorylates GST-fusion proteins of the major intracellular domains of $GABA_A$ receptor β and γ2 subunits, *in vitro* (McDonald and Moss, 1994, 1997). Further investigations using site-directed mutagenesis revealed a number of phosphorylation sites. These include S384 and S409 in the β1 subunit (McDonald and Moss, 1994), S410 in the β2 subunit, and S383 and S409 in the β3 subunit (McDonald and Moss, 1997). They also include S348 and T350, in the γ2S and γ2L subunits, as well as S343 in the γ2L subunit only (McDonald and Moss, 1994; Machu *et al.*, 1993). CaMKII has also been shown to phosphorylate the $GABA_C$ receptor subunit, ρ1, at T431, *in vitro* (Sedelnikova and Weiss, 2002). Finally, activation of CaMKII in a synaptosomal membrane fraction has been shown to cause an increase in phosphorylation of the $GABA_A$ receptor α1 subunit (Churn *et al.*, 2002), and to enhance agonist and allosteric modulator binding (Churn and DeLorenzo, 1998; Churn *et al.*, 2002).

Although $GABA_A$ receptors are binding-partners and *in vitro* substrates of CaMKII, it remains largely unknown whether $GABA_A$ receptors are substrates of CaMKII in

neurons. In this chapter, I have taken a biochemical-based approach using *in vitro* kinase and phosphatase assays, pre-labelling assays, affinity-purification assays and quantitative Western blotting to address this issue. I set the following aims: (1) to confirm and extend previous findings demonstrating that $GABA_A$ receptor β and γ2 subunits are *in vitro* substrates of CaMKII; (2) to identify protein phosphatases that dephosphorylate $GABA_A$ receptor subunits that have been phosphorylated by CaMKII, *in vitro*; (3) to determine whether phosphorylation-dependent targeting of CaMKII to receptor β3 subunits is accompanied by enhanced kinase activity, and (4) to examine the phosphorylation of $GABA_A$ receptors *in situ*, in cultured cortical neurons. This approach has led to the discovery that CaMKII selectively phosphorylates the $GABA_A$ receptor β and γ subunits, but not the α or δ subunits, *in vitro*. It has also shown that the γ1 and γ3 subunits are novel *in vitro* substrates of CaMKII, and that the β3 subunit is dephosphorylated at the CaMKII sites by PP1, PP2A and PP2C, *in vitro*. Depolarisation of cultured immature cortical neurons has also been found to enhance the level of enzymatically active CaMKII that associates with the major intracellular domain of the $GABA_A$ receptor β3 subunit, and to trigger CaMKII-dependent phosphorylation of the β3 subunit, but not the γ2 subunit, *in situ*. Since protein phosphorylation plays an important role in regulating $GABA_A$ receptor function and hence inhibitory synaptic transmission (Moss and Smart, 2001; Poisbeau *et al.*, 1999; Section 1.7.2), I propose that CaMKII-dependent phosphorylation of $GABA_A$ receptors is a major mechanism for modulating GABAergic synaptic transmission and neuronal excitability.

4.2 Results

An overview of the methods used in this chapter can be found in Figure 3.1.

4.2.1 CaMKII Phosphorylates $GABA_A$ Receptor Subunits, *In Vitro*

4.2.1.1 CaMKII Selectively Phosphorylates the Major Intracellular Domains of $GABA_A$ Receptor β and γ Subunits, In Vitro

To confirm that the major intracellular domains of $GABA_A$ receptor β and γ2 subunits are *in vitro* substrates of CaMKII (McDonald and Moss, 1994, 1997), I performed *in*

vitro kinase assays using purified CaMKII and purified, bacterially expressed GST-fusion proteins of the major intracellular domain of $GABA_A$ receptor β1, β2, β3, γ2S and γ2L subunits (GST-β1, GST-β2 GST-β3 GST-γ2S GST-γ2L) as well as recombinant GST (Section 2.2.4; Kittler *et al.*, 2005). GST-fusion proteins of the major intracellular domain of the γ1, γ3 and δ subunits (GST-γ1, GST-γ3 GST-δ) were also included in the assays to determine whether these subunits are *in vitro* substrates of CaMKII. The GST-fusion proteins were each incubated with CaMKII, which had been purified from brain (a generous gift from Howard Schulman) (Schulman, 1984), calmodulin, PKA and PKC inhibitors, and a [γ-^{32}P]-ATP/ATP mix, in CaMKII Buffer at 30 °C for 15 min (Section 2.5.13). After resolving the proteins by SDS-PAGE, phosphorylated proteins were detected by phosphorimaging. I obtained [^{32}P]-protein bands at the predicted molecular weight of the GST-fusion proteins tested, in assays using GST-β1 (~43 kDa), GST-β2 (~42 kDa), GST-β3 (~46 kDa), GST-γ1 (~34 kDa), GST-γ2S (~36 kDa), GST-γ2L (~38 kDa) and GST-γ3 (~42 kDa) (Fig. 4.1A) (McDonald and Moss, 1994, 1997). I did not detect any [^{32}P]-protein bands in assays using GST-α1, GST-δ or GST alone (~29 kDa) (Fig. 4.1A). The strongest [^{32}P]-protein band was obtained with GST-β3 (Fig. 4.1A). [^{32}P]-protein bands detected at lower molecular weights represented degradation products of the GST-fusion proteins (Fig. 4.1A). These results suggested that purified CaMKII selectively and directly phosphorylates the major intracellular domains of $GABA_A$ receptor β and γ subunits, but not α1 or δ subunits, *in vitro*, and that GST-β3 is the preferred *in vitro* substrate.

4.2.1.2 CaMKII Phosphorylates the Major Intracellular Domain of the $GABA_A$ Receptor β3 Subunit at S383 and S409, In Vitro

To confirm that S383 and S409 are the sites of CaMKII phosphorylation in GST-β3 (McDonald and Moss, 1997) (the preferred *in vitro* substrate of CaMKII), I performed *in vitro* kinase assays using purified CaMKII and purified, bacterially expressed GST-fusion proteins. These included wild type GST-β3, and mutant GST-β3-fusion proteins in which various serine residues were replaced with alanine residues (GST-β3-S408A, GST-β3-S409A, GST-β3-S408A-S409A, GST-β3-S383A-S408A-S409A) (McDonald and Moss, 1997). The wild type and mutant GST-fusion

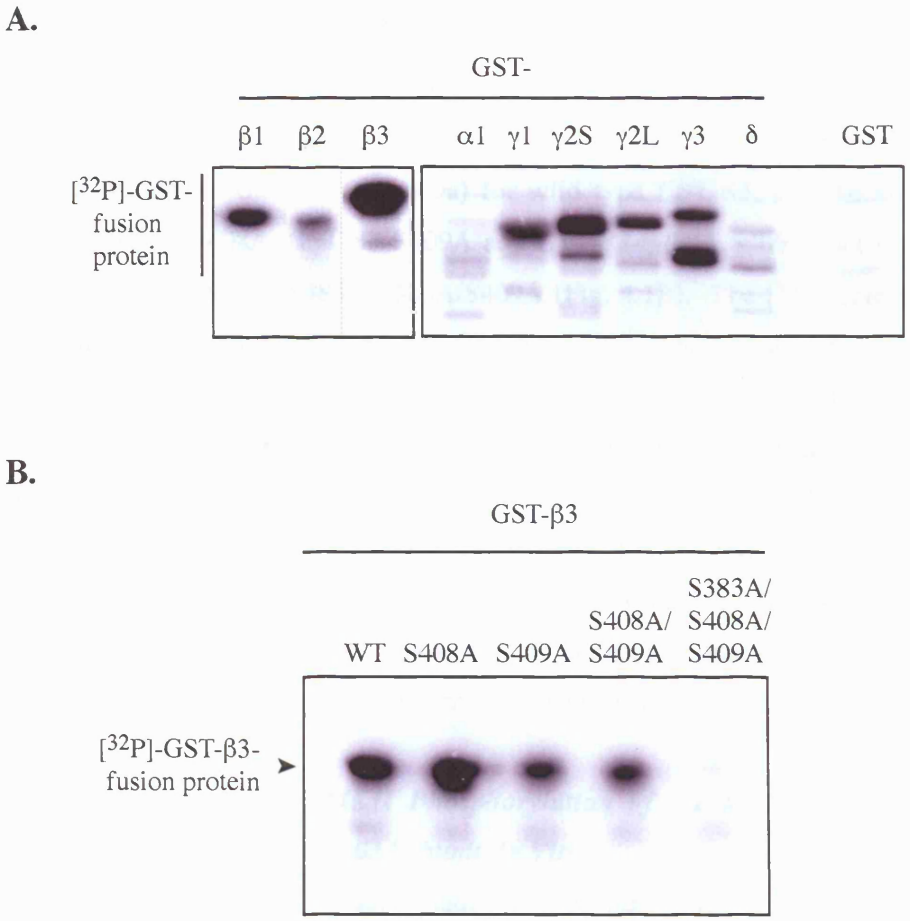


Figure 4.1. CaMKII phosphorylates various $GABA_A$ receptor subunits, *in vitro*. **A.** CaMKII selectively phosphorylates the major intracellular domains of $GABA_A$ receptor β and γ subunits. *In vitro* kinase assays were performed with purified CaMKII and GST-fusion proteins of the major intracellular domain of $GABA_A$ receptor α1 (3 μg), β1 (5 μg), β2 (5 μg), β3 (5 μg), γ1 (3 μg), γ2S (3 μg), γ2L (3 μg), γ3 (3 μg) and δ (3 μg) subunits, as well as GST alone (5 μg), at 30 °C for 15 min. Proteins were then resolved by SDS-PAGE, and phosphorylated proteins were detected by phosphorimaging. **B.** CaMKII phosphorylates the major intracellular domain of the $GABA_A$ receptor β3 subunit at S383 and S409. *In vitro* kinase assays were performed as in 'A.' but with 4 μg each of wild-type GST-β3, and the mutants GST-β3-S408A, GST-β3-S409A, GST-β3-S408A/S409A and GST-β3-S383A/S408A/S409A. Proteins were then resolved by SDS-PAGE, and phosphorylated proteins were detected by phosphorimaging.

proteins were each incubated with purified CaMKII, calmodulin, PKA and PKC inhibitors and a [γ-^{32}P]-ATP/ATP mix, in CaMKII Buffer at 30 °C for 15 min. All proteins were then resolved by SDS-PAGE, and phosphorylated proteins were detected by phosphorimaging. I detected [^{32}P]-protein bands at the predicted molecular weight of GST-β3 (~46 kDa) for wild type GST-β3, and the GST-β3 mutants, GST-β3-S408A, GST-β3-S409A and GST-β3-S408A/S409A, but not with the triple mutant GST-β3-S383A/S408A/S409A (Fig. 4.1B). The [^{32}P]-protein band was stronger for wild type GST-β3 and GST-β3-S408A, which contain both sites of phosphorylation, than for GST-β3-S409A and GST-β3-S408A/S409A, which contain only one of the phosphorylation sites, S383 (Fig. 4.1B). These data demonstrated that wild type GST-β3, and the GST-β3 mutants, GST-β3-S408A, GST-β3-S409A and GST-β3-S408A/S409A, but not GST-β3-S383A/S408A/S409A, were *in vitro* substrates of purified CaMKII, and that GST-β3-S409A and GST-β3-S408A/S409A were the weakest substrates. These results suggested that purified CaMKII phosphorylates the major intracellular domain of the β3 subunit at S383 and S409.

4.2.1.3 Time-Course of CaMKII Phosphorylation of the Major Intracellular domain of the $GABA_A$ Receptor β3 Subunit, In Vitro

I next analysed the *in vitro* phosphorylation of wild type GST-β3 by CaMKII with respect to time. GST-β3 (5 μg) was incubated with CaMKII, which had been purified from brain (a generous gift from Paul Greengard) (Nairn and Greengard, 1987), calmodulin, and a [γ-^{32}P]-ATP/ATP mix, in CaMKII Buffer at 30 °C for 1, 2, 5, 10, 20, 30 and 60 min (Section 2.5.13). After resolving the proteins by SDS-PAGE, phosphorylated proteins were detected and quantitated by phosphorimaging. I obtained a [^{32}P]-protein band at the predicted molecular weight of GST-β3 (~46 kDa) and the autophosphorylated 50- and 60-kDa subunits of CaMKII in each kinase assay (Fig. 4.2A and B). The intensity of the [^{32}P]-GST-β3 band increased with time (Fig. 4.2A and B), and the maximum stoichiometry of phosphorylation (~0.22 mol. phosphate mol. protein^{-1}) was attained within 20 min (Fig. 4.2C). The rapid and robust phosphorylation of GST-β3 by CaMKII suggested that the major intracellular domain of the $GABA_A$ receptor β3 subunit is a good *in vitro* substrate of purified

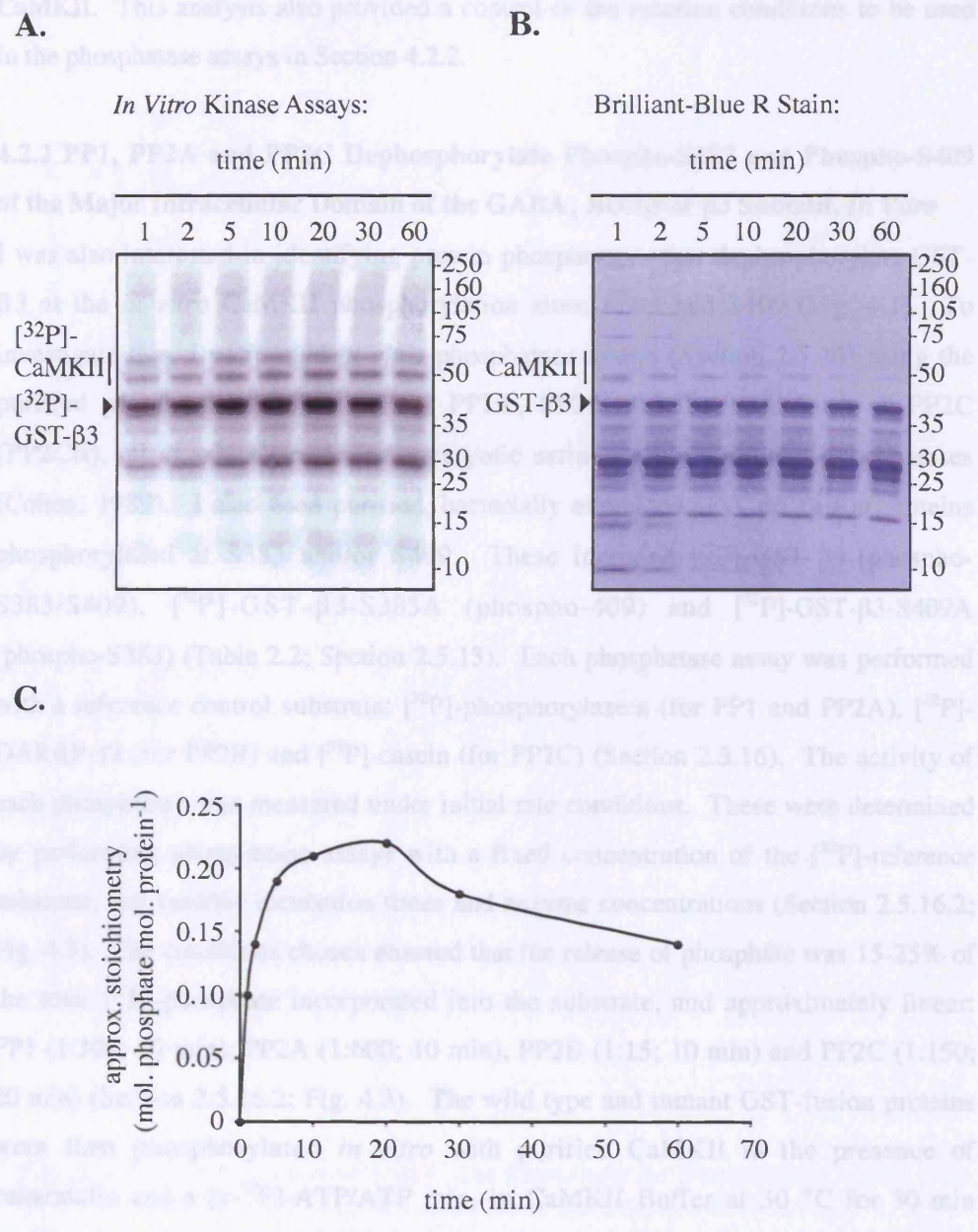


Figure 4.2. Time-course showing the *in vitro* phosphorylation of GST-β3 by CaMKII. A. GST-β3 (5 μg) fusion proteins were phosphorylated *in vitro* using purified CaMKII and [γ-^{32}P]-ATP for the indicated times at 30 °C. Proteins were then resolved by SDS-PAGE, and [^{32}P]-GST-fusion proteins were detected by phosphorimaging. **B.** Brilliant Blue-R stain of the gel in 'A.'. **C.** The stoichiometry of phosphorylation was assessed after SDS-PAGE and phosphorimaging.

CaMKII. This analysis also provided a control of the reaction conditions to be used in the phosphatase assays in Section 4.2.2.

4.2.2 PP1, PP2A and PP2C Dephosphorylate Phospho-S383 and Phospho-S409 of the Major Intracellular Domain of the $GABA_A$ Receptor β3 Subunit, *In Vitro*

I was also interested in identifying protein phosphatases that dephosphorylate GST-β3 at the *in vitro* CaMKII phosphorylation sites, S383 and S409 (Fig. 4.1). To investigate this, I performed *in vitro* phosphatase assays (Section 2.5.16) using the purified protein phosphatases, PP1, PP2A, PP2B and the α isoform of PP2C (PP2Cα), all of which are major eukaryotic serine/threonine-protein phosphatases (Cohen, 1989). I also used purified, bacterially expressed GST-β3 fusion proteins phosphorylated at S383 and/or S409. These included [^{32}P]-GST-β3 (phospho-S383/S409), [^{32}P]-GST-β3-S383A (phospho-409) and [^{32}P]-GST-β3-S409A (phospho-S383) (Table 2.2; Section 2.5.13). Each phosphatase assay was performed with a reference control substrate: [^{32}P]-phosphorylase a (for PP1 and PP2A), [^{32}P]-DARRP-32 (for PP2B) and [^{32}P]-casein (for PP2C) (Section 2.5.16). The activity of each phosphatase was measured under initial rate conditions. These were determined by performing phosphatase assays with a fixed concentration of the [^{32}P]-reference substrate, and variable incubation times and enzyme concentrations (Section 2.5.16.2; Fig. 4.3). The conditions chosen ensured that the release of phosphate was 15-25% of the total [^{32}P]-phosphate incorporated into the substrate, and approximately linear: PP1 (1:300; 10 min), PP2A (1:600; 10 min), PP2B (1:15; 10 min) and PP2C (1:150; 20 min) (Section 2.5.16.2; Fig. 4.3). The wild type and mutant GST-fusion proteins were then phosphorylated *in vitro* with purified CaMKII in the presence of calmodulin and a [γ-^{32}P]-ATP/ATP mix, in CaMKII Buffer at 30 °C for 30 min (Section 2.5.13). A sample of each kinase assay was resolved by SDS-PAGE, and phosphorylated proteins were detected by phosphorimaging. I detected [^{32}P]-protein bands at the predicted molecular weight of each of the GST-β3 fusion proteins (~46 kDa) (Fig. 4.4A). Although [^{32}P]-protein bands were also detected at the predicted molecular weight of the autophosphorylated 50- and 60-kDa subunits of CaMKII, these contributed only a small proportion of the total [^{32}P]-signals (Fig. 4.4A).

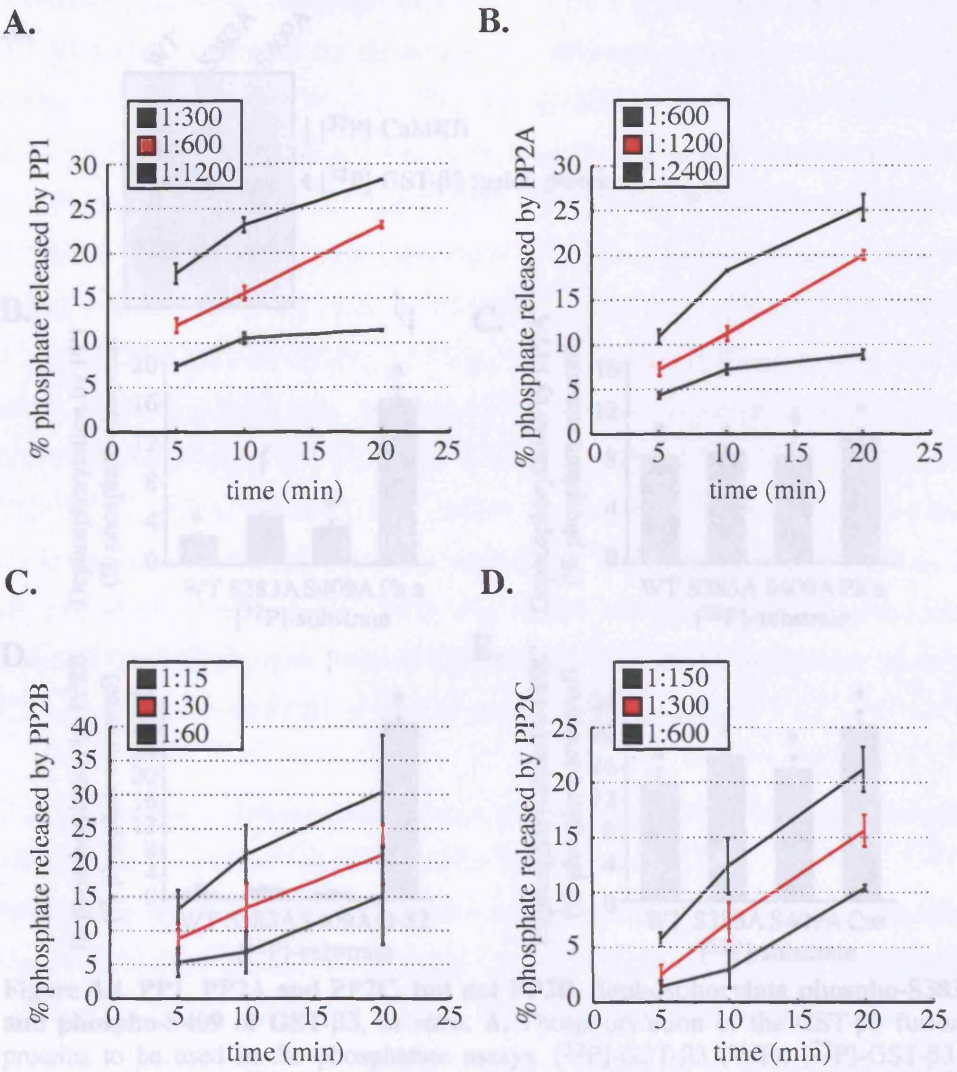


Figure 4.3. Determination of initial rate conditions for phosphatase assays with PP1, PP2A, PP2B and PP2C. The activities of the indicated dilutions of PP1, PP2A, PP2B and PP2C were measured at 30 °C for the indicated time (min) using a [^{32}P]-labelled control substrate (~0.5 μM). The [^{32}P]-substrates were also incubated in the absence of phosphatase as a control. The amount of [^{32}P] released (cpm) in the presence of phosphatase in the indicated time was expressed as a percentage of the total [^{32}P] input (cpm). The data represents the rate of dephosphorylation (% phosphate removed) from at least 3 independent experiments (mean ± sem), which were each done in duplicate. **A.** PP1 and **B.** PP2A activity was assayed using [^{32}P]-phosphorylase a as a standard. **C.** PP2B activity was assayed using [^{32}P]-DARRP-32-T34 as a standard. **D.** PP2Cα activity was assayed using [^{32}P]-casein as a standard. cpm: counts min^{-1}.

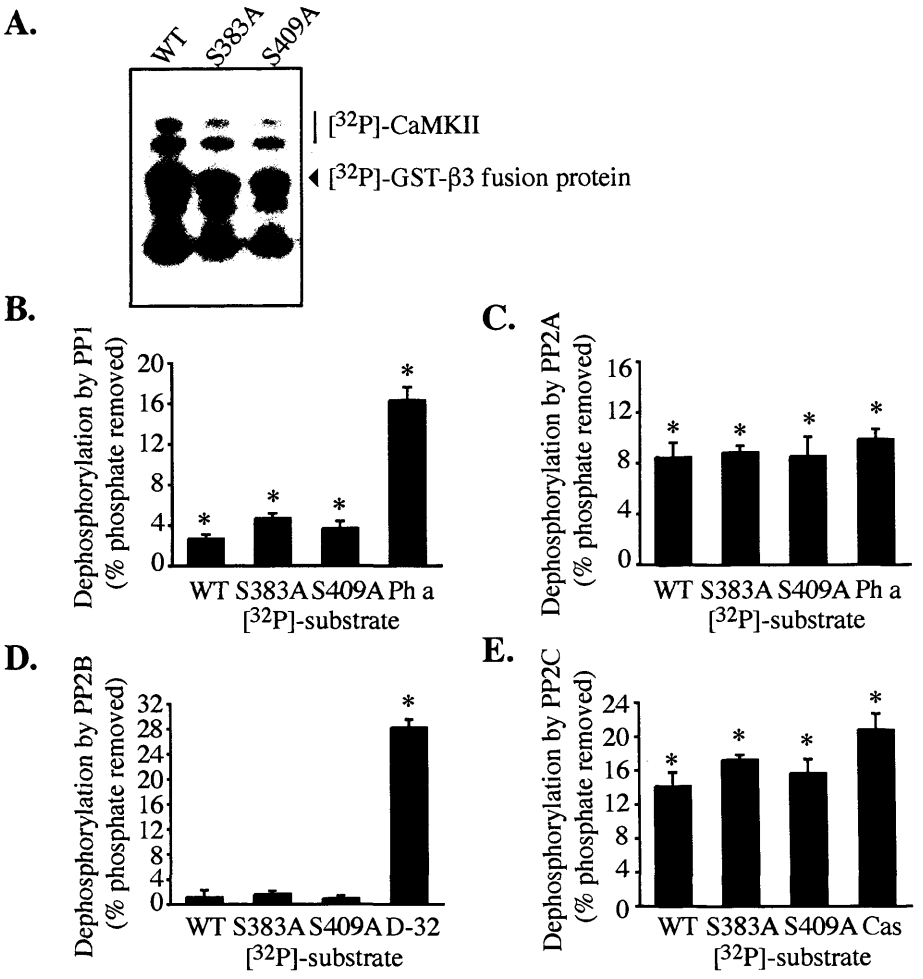


Figure 4.4. PP1, PP2A and PP2C, but not PP2B, dephosphorylate phospho-S383 and phospho-S409 of GST-β3, *in vitro*. **A.** Phosphorylation of the GST-β3 fusion proteins to be used in the phosphatase assays. [^{32}P]-GST-β3 (WT), [^{32}P]-GST-β3-S383A (S383A) and [^{32}P]-GST-β3-S409A (S409A) were each phosphorylated *in vitro* by purified CaMKII at 30 °C for 15 min. A sample of each reaction was separated by SDS-PAGE and [^{32}P]-proteins were visualised by phosphorimaging. **B-E.** The activities of purified protein phosphatases were measured under initial rate conditions using each [^{32}P]-GST-β3 fusion protein (~0.5 μM; dark grey bars), and a [^{32}P]-reference substrate (~0.5 μM; light grey bars). The amount of [^{32}P] released (cpm) in the absence of phosphatase was subtracted from that released in the presence of phosphatase, and the resultant value was expressed as a percentage of the total [^{32}P] incorporated (cpm). The rates of dephosphorylation (% phosphate removed) were determined from at least 3 independent experiments, each done in duplicate, and plotted as shown (mean ± sem). Dephosphorylation of each test and reference substrate was compared to baseline (0%) using the Student's paired *t* test. * denotes $p<0.05$. **B.** PP1 **C.** PP2A and **D.** PP2B activities were assayed for 10 min, and **E.** PP2C, for 20 min. cpm: counts min^{-1}, Ph a: [^{32}P]-phosphorylase a; D-32: [^{32}P]-DARRP-32-T34, Cas: [^{32}P]-casein.

As shown in Figure 4.4 (B, C and E), [^{32}P]-GST-β3, [^{32}P]-GST-β3-S383A and [^{32}P]-GST-β3-S409A, as well as the respective [^{32}P]-reference substrates, were substrates of PP1 ([^{32}P]-GST-β3: 2.7%±0.4; [^{32}P]-GST-β3-S383A: 4.7%±0.5; [^{32}P]-GST-β3-S409A: 3.7%±0.7; [^{32}P]-phosphorylase a: 16.3%±1.3; n=3; $p<0.05$, Student's paired *t* test), PP2A ([^{32}P]-GST-β3: 8.5%±1.2; [^{32}P]-GST-β3-S383A: 8.8%±0.5; [^{32}P]-GST-β3-S409A: 8.5%±1.5; [^{32}P]-phosphorylase a: 9.9%±0.8; n=at least 3; $p<0.05$, Student's paired *t* test) and PP2C ([^{32}P]-GST-β3: 14.2%±1.7; [^{32}P]-GST-β3-S383A: 17.3%±0.6; [^{32}P]-GST-β3-S409A: 15.7%±1.7; [^{32}P]-casein: 20.8%±1.9; n=at least 3; $p<0.05$, Student's paired *t* test). In contrast, PP2B did not dephosphorylate any of the GST-β3 fusion proteins ([^{32}P]-GST-β3: 1.1%±1.1; [^{32}P]-GST-β3-S383A: 1.6%±0.6; [^{32}P]-GST-β3-S409A: 0.9%±0.5; n=3; $p>0.05$, Student's paired *t* test), despite being able to dephosphorylate the reference substrate, [^{32}P]-DARRP-32-T34 (28.2%±1.3; n=3; $p<0.05$, Student's paired *t* test) (Fig. 4.4D). These results suggested that phospho-S383 and phospho-S409 of GST-β3 are each dephosphorylated by PP1, PP2A and PP2C, but not PP2B, in these *in vitro* phosphatase assays.

4.2.3 KCl-Induced Depolarisation of Cultured Immature Cortical Neurons Enhances the Functional Interaction of CaMKII with GST-β3

I have previously shown that CaMKII phosphorylates GST-β3 fusion proteins *in vitro* (Figs. 4.1 and 4.2), and that recombinant CaMKIIα binds to the major intracellular domain of the $GABA_A$ receptor β subunits only when in the constitutively active form (CaMKIIα-T286D) (Fig. 3.3). I was therefore interested in examining whether depolarisation of neurons and physiological activation of endogenous CaMKII increases the binding of enzymatically active CaMKII to GST-β3. To investigate this, I used a combination of neuronal treatments, affinity-purification assays and *in vitro* kinase assays (Section 2.5.15; Fig. 3.1), which will be collectively referred to as 'pull-down'-kinase assays from hereon, and purified, bacterially expressed GST-β3 and recombinant GST fusion proteins. To perform the 'pull-down'-kinase assays, primary cultures of cortical neurons (E17; 6-8DIV) were incubated in the absence or presence of KCl (50 mM) with or without the CaMKII inhibitor KN93 (4 μM) for 5 min at 37 °C (in HEPES-buffered saline (HBS); Section 2.5.12). KN93 competes with calmodulin to inhibit autophosphorylation of CaMKII (Sumi *et al.*, 1991), and

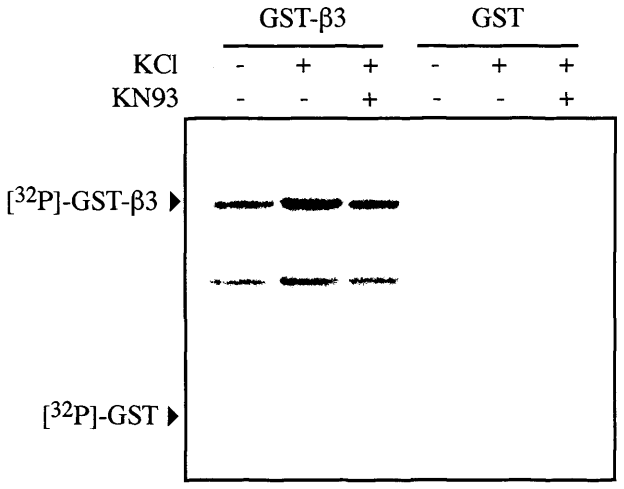
was added 20 min before the KCl incubation to enable it to enter the neurons and exert its effect. Following the treatments, the neurons were lysed under non-denaturing conditions, and the neuronal extracts were used in affinity-purification assays with GST-β3 or GST alone. Following extensive washing, the precipitates were re-suspended in CaMKII Buffer (which does not contain calmodulin) and *in vitro* kinase assays were performed in the presence of [γ-^{32}P]-ATP at 30 °C for 30 min. After resolving the proteins by SDS-PAGE, [^{32}P]-GST-β3 bands were detected and quantitated by phosphorimaging, and normalised to the untreated control.

As shown in Figure 4.5A, I detected [^{32}P]-protein bands at the predicted molecular weight of GST-β3 (~46 kDa) from all 'pull-down'-kinase assays using GST-β3. I did not detect any [^{32}P]-protein bands in the assays performed using GST alone (Fig. 4.5A). I obtained a stronger [^{32}P]-GST-β3 band in assays that were performed using extracts of KCl-treated neurons (146.9±13.2%; $p<0.05$; Student's paired *t* test; n=4) compared to control neurons (100%) (Fig. 4.5). However, a significant enhancement in phosphorylation of GST-β3 was not observed when the neurons were pre-treated with KN93 (113.6±18.2%; $p>0.05$; Student's paired *t* test; n=3) (Fig. 4.5). These data demonstrated that depolarisation of cultured immature cortical neurons by KCl increased the level of KN93-sensitive kinase activity that bound to GST-β3. This suggested that depolarisation of cultured immature cortical neurons enhances the level of enzymatically active CaMKII that binds and phosphorylates GST-β3.

4.2.4 Depolarisation-Induced Autophosphorylation of CaMKII at T286 in Cultured Immature Cortical Neurons

I was next interested in investigating CaMKII activity in neurons with a view to examining CaMKII-dependent phosphorylation of $GABA_A$ receptors, *in situ*. $GABA_A$ receptors mediate depolarising responses during neuronal development (Section 1.2), and previous studies have shown that activation of $GABA_A$ receptors triggers depolarisation and calcium influx in primary cultures of immature cortical neurons (E17; 6DIV; Section 2.4.2) (J.N. Jovanovic, unpublished data). I was therefore interested in determining whether stimulation of $GABA_A$ receptors triggers autophosphorylation and activation of CaMKII in this neuronal preparation.

A.



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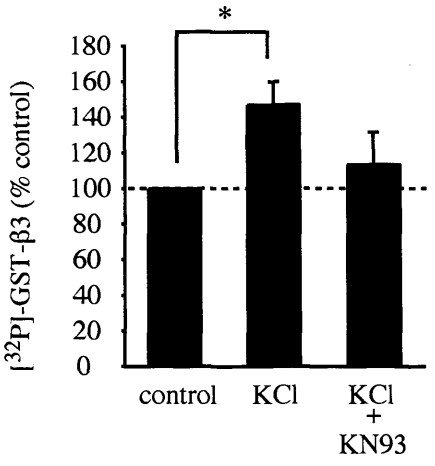


Figure 4.5. Depolarisation of immature cortical neurons enhances the functional interaction of neuronal CaMKII with GST-β3. Primary cultures of cortical neurons (E17; 6-8DIV) were incubated in the absence (-) or presence (+) of KCl (50 mM) with (+) or without (-) KN93 (4 μM; added 20 min prior to treatment) for 5 min at 37 °C. Neurons were then lysed under non-denaturing conditions, and the extracts were used in an affinity-purification assay with GST-β3 or GST. A kinase assay was then performed in CaMKII buffer in the presence of [γ-^{32}P]-ATP at 30 °C for 30 min and proteins were resolved by SDS-PAGE. **A.** Visualisation of [^{32}P]-GST-fusion proteins by phosphorimaging. **B.** Quantitation of [^{32}P]-GST-β3 protein bands from 'A.' (mean ± sem; n = 3 or above; Student's paired *t* test; * denotes $p<0.05$).

4.2.4.1 Basal Activity of CaMKII in Cultured Immature Cortical Neurons

I began by examining the level of basal CaMKII activity in primary cultures of immature cortical neurons (E17; 6DIV). To do this, neurons were incubated for 5 min at 37 °C in the absence or presence of the CaMKII inhibitor, KN93 (4 μM), and the calcium (intracellular and extracellular) chelator, BAPTA-AM (25 μM) (in HBS). KN93 and BAPTA-AM were added 20 min and 5 min, respectively, before the 5 min incubation to enable them to enter the neurons and exert their effect. Following the incubations, the neurons were lysed under denaturing conditions, and equal amounts of protein were resolved by SDS-PAGE and analysed by quantitative Western blotting and phosphorimaging. Phosphorylation of endogenous CaMKIIα at T286 was detected using a polyclonal anti-CaMKII-pT286 phosphorylation state-specific antibody (Table 2.6). Total CaMKIIα protein levels were also examined using a monoclonal anti-CaMKIIα antibody.

As shown in Figure 4.6, I detected an immunoreactive band at the predicted molecular weight (50 kDa) of CaMKIIα-pT286 (Fig. 4.6A) and CaMKIIα (Fig. 4.6C) from the control and treated neurons. I performed a quantitative analysis of these findings by measuring the intensity of the band from each set of treated neurons, and normalising it to the value obtained from the untreated control neurons. The results from several experiments (mean ± sem; n=3-7) were compared with the untreated control using the Student's paired *t* test (Fig. 4.6B and D). This analysis revealed a statistically significant decrease in the basal level of CaMKIIα-T286 phosphorylation in neurons that were treated with KN93 (63.3±13.2; $p<0.05$; Student's paired *t* test; n=7) or BAPTA-AM (64.7±4.0; $p<0.05$; Student's paired *t* test; n=4) (Fig. 4.6B). I did not detect a statistically significant change in the total level of CaMKIIα protein in neurons treated with KN93 (91.7±11.1; $p>0.05$; Student's paired *t* test; n=5) (Fig. 4.6D). However, a statistically significant decrease in the total level of CaMKIIα protein was detected in neurons that were treated with BAPTA-AM (75.6±5.2; $p<0.05$; Student's paired *t* test; n=3) (Fig. 4.6D). These data demonstrated that CaMKIIα is basally phosphorylated at T286 in cultured immature cortical neurons, and that the basal level of T286-phosphorylation is sensitive to the CaMKII inhibitor, KN93, and the calcium chelator, BAPTA-AM. The results also demonstrated that

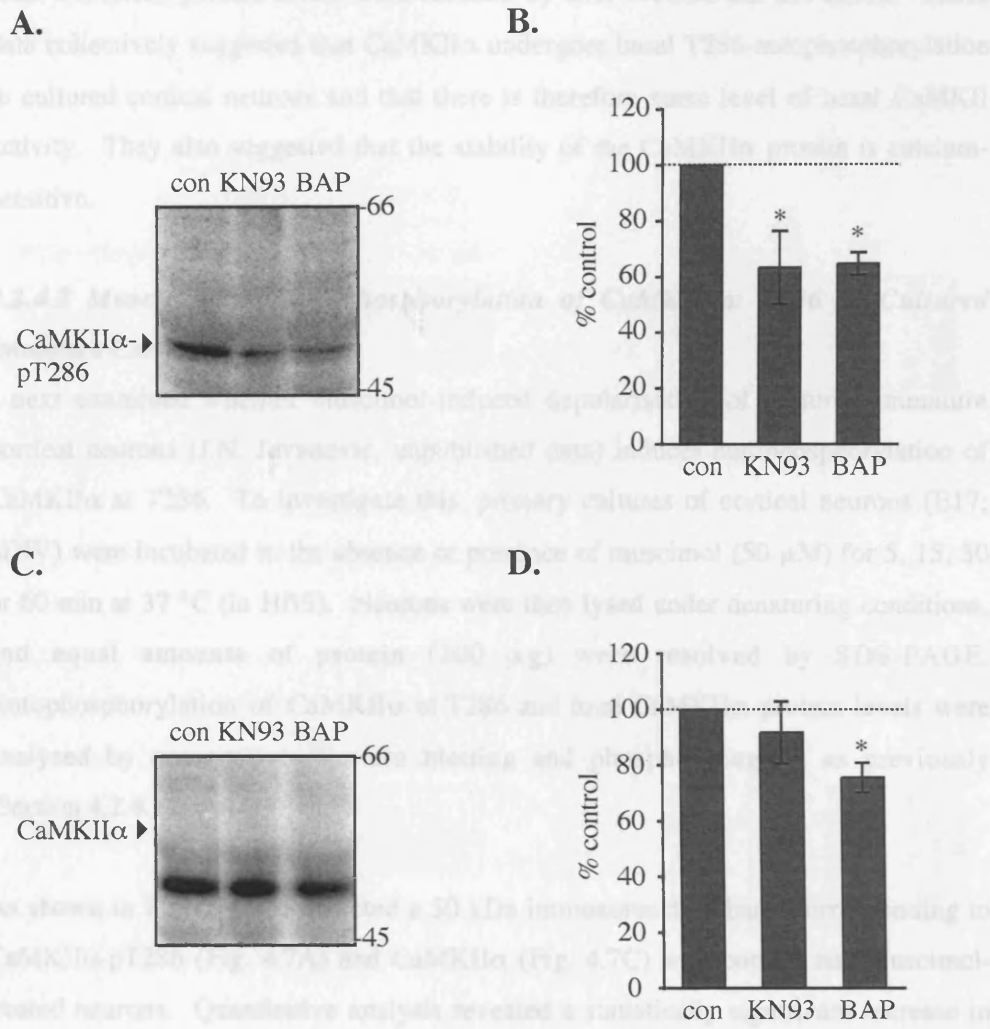


Figure 4.6. CaMKIIα is basally phosphorylated at T286 in immature cortical neurons. Primary cultures of cortical neurons (E17; 6DIV) were incubated at 37 ^{o}C for 5 min in the absence (con) or presence of the CaMKII inhibitor, KN93 (4 μM; 20 min pre-incubation), or the calcium chelator, BAPTA-AM (BAP; 25 μM; 5 min pre-incubation). Neurons were lysed under denaturing conditions and equal amounts of protein were separated by SDS-PAGE and analysed by quantitative Western blotting. **A.** Autophosphorylation of CaMKIIα at T286 (CaMKIIα-pT286) was analysed by probing cortical extracts (300 μg of protein) with a polyclonal anti-pT286 antibody and an [^{125}I]-anti-rabbit antibody. **B.** Quantitation of 'A.'. The data from each set of drug-treated neurons was normalised to that of the untreated control neurons. The results for a number of experiments (mean ± sem; n = 3-7) were then compared to the control group using the Student's paired *t* test (* denotes $p<0.05$). **C.** Total CaMKIIα protein levels were examined by immunoblotting cortical extracts (100 μg of protein) with a monoclonal anti-CaMKIIα antibody (CaMKIIα), a rabbit anti-mouse bridge antibody and an [^{125}I]-anti-rabbit antibody. **D.** Quantitation of 'C.', as for 'B.'.

total CaMKIIα protein levels were reduced by BAPTA-AM but not KN93. These data collectively suggested that CaMKIIα undergoes basal T286-autophosphorylation in cultured cortical neurons and that there is therefore some level of basal CaMKII activity. They also suggested that the stability of the CaMKIIα protein is calcium-sensitive.

4.2.4.2 Muscimol-Induced Phosphorylation of CaMKII at T286 in Cultured Immature Cortical Neurons

I next examined whether muscimol-induced depolarisation of cultured immature cortical neurons (J.N. Jovanovic, unpublished data) induces autophosphorylation of CaMKIIα at T286. To investigate this, primary cultures of cortical neurons (E17; 6DIV) were incubated in the absence or presence of muscimol (50 μM) for 5, 15, 30 or 60 min at 37 °C (in HBS). Neurons were then lysed under denaturing conditions, and equal amounts of protein (300 μg) were resolved by SDS-PAGE. Autophosphorylation of CaMKIIα at T286 and total CaMKIIα protein levels were analysed by quantitative Western blotting and phosphorimaging, as previously (Section 4.2.4.1).

As shown in Figure 4.7, I detected a 50-kDa immunoreactive band corresponding to CaMKIIα-pT286 (Fig. 4.7A) and CaMKIIα (Fig. 4.7C) with control and muscimol-treated neurons. Quantitative analysis revealed a statistically significant increase in phosphorylation of CaMKIIα-T286 above control levels following incubation with muscimol for 5 min (135.1±8.1; $p<0.05$; Student's paired t test; n=9) and 15 min (128.0±6.0; $p<0.05$; Student's paired t test; n=5), but not 30 min (118.4±9.1; $p>0.05$; Student's paired t test; n=5) or 60 min (99.6±12.4; $p>0.05$; Student's paired t test; n=6) (Fig. 4.7B). I did not detect a statistically significant change in the total level of CaMKIIα protein after treatment with muscimol for 5 min (105.9±8.2; $p>0.05$; Student's paired t test; n=7), 15 min (109.0±10.4; $p>0.05$; Student's paired t test; n=4), 30 min (106.7±8.7; $p>0.05$; Student's t test; n=5) or 60 min (100.4±14.9; $p>0.05$; Student's paired t test; n=6) (Fig. 4.7D). These data demonstrated a statistically significant increase in phosphorylation of CaMKIIα at T286 above control levels in cultured immature cortical neurons following incubation with

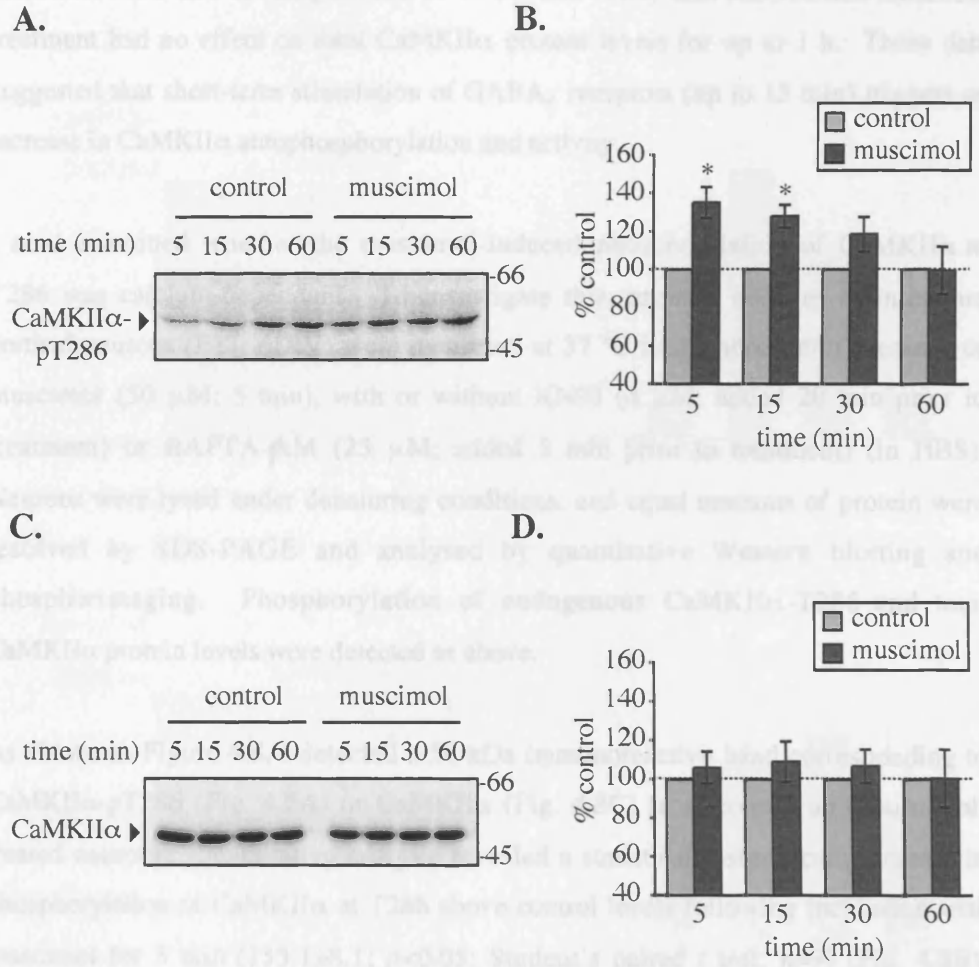


Figure 4.7. Time-course of muscimol-induced phosphorylation of CaMKIIα-T286 in immature cortical neurons. Primary cultures of cortical neurons (E17; 6DIV) were incubated at 37 ^{O}C for the indicated time (min) in the absence (control) or presence of muscimol (50 μM). Neurons were lysed under denaturing conditions, and equal amounts of protein were resolved by SDS-PAGE and analysed by quantitative Western blotting. **A.** Phosphorylation of CaMKIIα at T286 (CaMKIIα-pT286) was analysed by probing cortical extracts (300 μg of protein) with a polyclonal anti-CaMKII-pT286 antibody and an [^{125}I]-anti-rabbit antibody. **B.** Quantitation of 'A.'. The band intensity measured from each muscimol-treated group was normalised to the untreated control value. The results of several experiments (mean ± sem; n = 4-9) were compared with the untreated control using the Student's paired *t* test (* denotes $p<0.05$). **C.** Total CaMKIIα protein levels (CaMKIIα) were examined by immunoblotting extracts (100 μg of protein) with a monoclonal anti-CaMKIIα antibody, a rabbit anti-mouse bridging antibody and an [^{125}I]-anti-rabbit antibody. **D.** Quantitation of 'C.', as for 'B.'.

muscimol for 5 and 15 min, but not 30 or 60 min. They also showed that muscimol treatment had no effect on total CaMKIIα protein levels for up to 1 h. These data suggested that short-term stimulation of $GABA_A$ receptors (up to 15 min) triggers an increase in CaMKIIα autophosphorylation and activity.

I next examined whether the muscimol-induced phosphorylation of CaMKIIα at T286 was calcium-dependent. To investigate this, primary cultures of immature cortical neurons (E17; 6DIV) were incubated at 37 °C in the absence or presence of muscimol (50 μM; 5 min), with or without KN93 (4 μM; added 20 min prior to treatment) or BAPTA-AM (25 μM; added 5 min prior to treatment) (in HBS). Neurons were lysed under denaturing conditions, and equal amounts of protein were resolved by SDS-PAGE and analysed by quantitative Western blotting and phosphorimaging. Phosphorylation of endogenous CaMKIIα-T286 and total CaMKIIα protein levels were detected as above.

As shown in Figure 4.8, I detected a 50-kDa immunoreactive band corresponding to CaMKIIα-pT286 (Fig. 4.8A) or CaMKIIα (Fig. 4.8C) from control and muscimol-treated neurons. Quantitative analysis revealed a statistically significant increase in phosphorylation of CaMKIIα at T286 above control levels following incubation with muscimol for 5 min (135.1±8.1; $p<0.05$; Student's paired *t* test; n=9) (Fig. 4.8B). This was not detected when neurons were also treated with KN93 (103.6±4.2; $p>0.05$; Student's paired *t* test; n=5), or BAPTA-AM, which caused a statistically significant decrease in autophosphorylation of CaMKIIα-T286 from control levels (69.3±10.9; $p<0.05$; Student's paired *t* test; n=5) (Fig. 4.8B). The analysis also revealed a statistically significant difference in the level of phosphorylation between neurons treated with muscimol, and neurons treated with muscimol and KN93 ($p<0.05$; Student's paired *t* test; n=5), or muscimol and BAPTA-AM ($p<0.05$; Student's paired *t* test; n=5) (Fig. 4.8B). I did not detect any statistically significant change from control in the level of total CaMKIIα protein following treatment with muscimol (105.9±8.2; $p>0.05$; Student's paired *t* test; n=7), muscimol with KN93 (116.5±8.3; $p>0.05$; Student's paired *t* test; n=5) or muscimol with BAPTA-AM (91.2±10.8; $p>0.05$; Student's paired *t* test; n=5) (Fig. 4.8D). These data

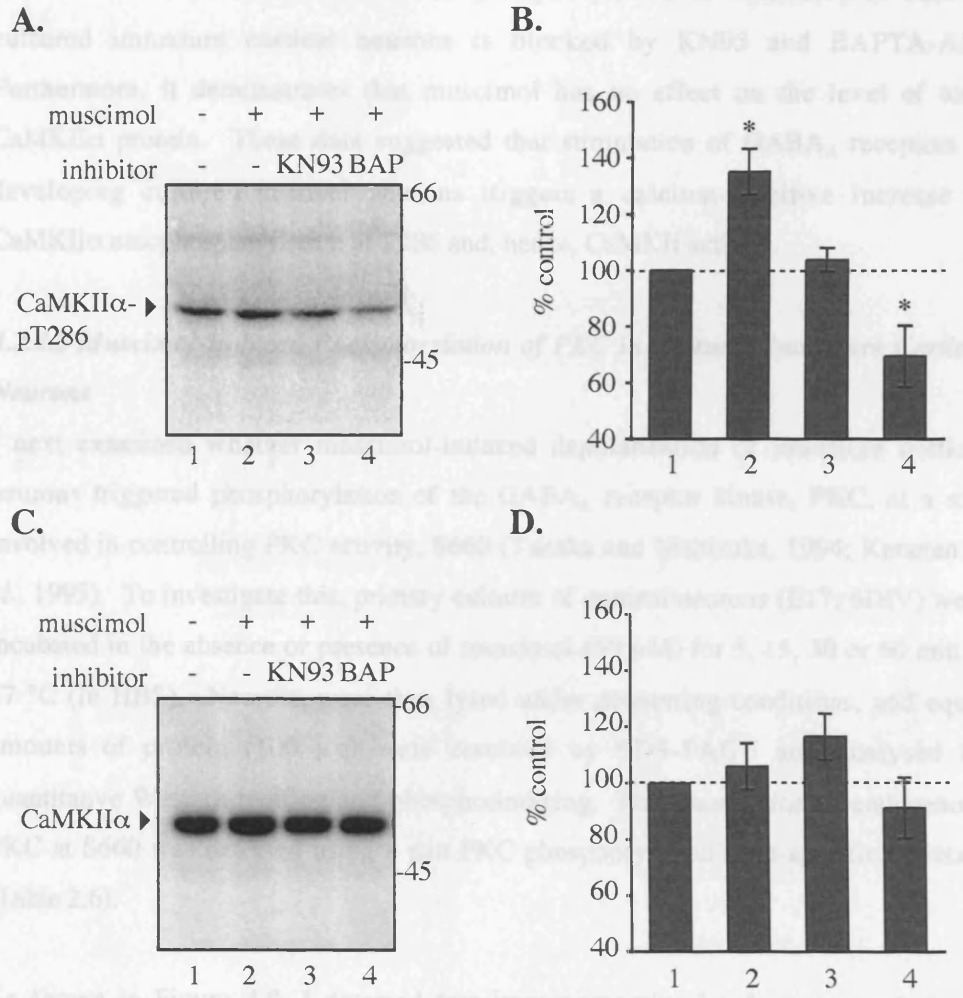


Figure 4.8. Muscimol-induced phosphorylation of CaMKIIα at T286 in immature cortical neurons is calcium sensitive. Primary cultures of cortical neurons (E17; 6DIV) were incubated at 37 ^{o}C for 5 min with (+; lanes 2-4) or without (-; lane 1) muscimol (50 μM) in the absence or presence of KN93 (4 μM; added 20 min prior to treatment; lane 3), or BAPTA-AM (BAP; 25 μM; added 5 min prior to treatment; lane 4). Neurons were lysed under denaturing conditions and equal amounts of protein were separated by SDS-PAGE and analysed by quantitative Western blotting. **A.** Phosphorylation of CaMKIIα at T286 (CaMKIIα-pT286) was analysed by probing cortical extracts (300 μg of protein) with a polyclonal anti-CaMKII-pT286 antibody and an [^{125}I]-anti-rabbit antibody. **B.** Quantitation of 'A.'. Data from each drug-treated group was normalised to the untreated control. The results from each drug-treated group from several experiments (mean ± sem; n = 5-9) were then compared to the control group using the Student's paired *t* test (* denotes $p<0.05$). **C.** Total CaMKIIα (CaMKIIα) protein levels were examined by immunoblotting extracts (100 μg of protein) with a monoclonal anti-CaMKIIα antibody, a rabbit anti-mouse bridging antibody and an [^{125}I]-anti-rabbit antibody. **D.** Quantitation of 'C.', as with 'B.'.

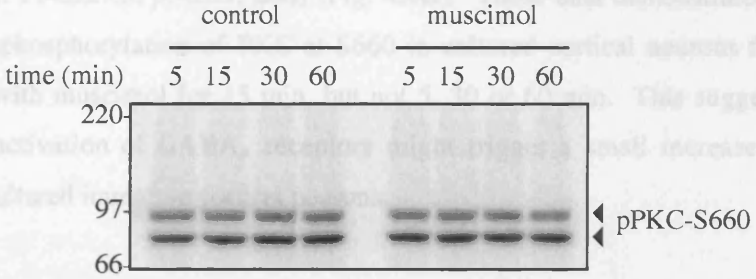
demonstrated that muscimol-induced phosphorylation of CaMKIIα at T286 in cultured immature cortical neurons is blocked by KN93 and BAPTA-AM. Furthermore, it demonstrates that muscimol has no effect on the level of total CaMKIIα protein. These data suggested that stimulation of $GABA_A$ receptors in developing cultured cortical neurons triggers a calcium-sensitive increase in CaMKIIα autophosphorylation at T286 and, hence, CaMKII activity.

4.2.4.3 Muscimol-Induced Phosphorylation of PKC in Cultured Immature Cortical Neurons

I next examined whether muscimol-induced depolarisation of immature cortical neurons triggered phosphorylation of the $GABA_A$ receptor kinase, PKC, at a site involved in controlling PKC activity, S660 (Tanaka and Nishizuka, 1994; Keranen *et al.*, 1995). To investigate this, primary cultures of cortical neurons (E17; 6DIV) were incubated in the absence or presence of muscimol (50 μM) for 5, 15, 30 or 60 min at 37 °C (in HBS). Neurons were then lysed under denaturing conditions, and equal amounts of protein (100 μg) were resolved by SDS-PAGE and analysed by quantitative Western blotting and phosphorimaging. Phosphorylation of endogenous PKC at S660 was detected using a pan PKC phosphorylation state-specific antibody (Table 2.6).

As shown in Figure 4.9, I detected two immunoreactive bands from control and muscimol-treated neurons: an upper band at 97 kDa and a lower band between 97 and 66 kDa, both of which are within the molecular weight range of the different PKC isoforms. I performed a quantitative analysis of these findings by measuring the intensity of the bands in each muscimol-treated group, and normalising each one to the value obtained from the respective untreated control. The results of several experiments (mean ± sem; n=5-8) were compared with the untreated control in the Student's paired *t* test (Fig. 4.9B). I detected a small but significant increase in phosphorylation of PKC at S660 following incubation with muscimol for 15 min (upper band: 114.9±4.9; $p<0.05$; n=6) (lower band: 111.0±2.8; $p<0.05$; n=6) (Fig. 4.9B). I did not detect a significant change in the level of phospho-PKC-S660 after treatment with muscimol for 5 min (upper band: 115.3±8.9; $p>0.05$; n=7) (lower

A.



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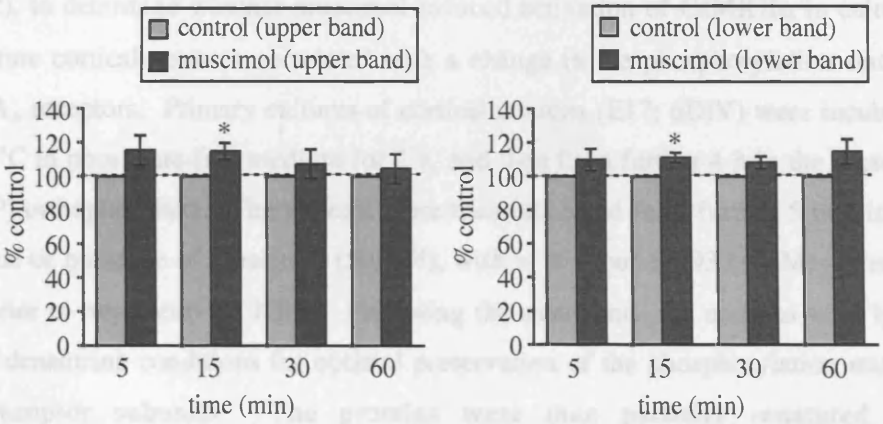


Figure 4.9. Time-course of PKC phosphorylation following treatment of immature cortical neurons with muscimol. Primary cultures of cortical neurons (E17; 6 DIV) were incubated at 37 ^{O}C for the indicated time (min) in the absence (control) or presence of muscimol (50 μM). Neurons were lysed under denaturing conditions, and equal amounts of protein (100 μg) were resolved by SDS-PAGE and analysed by quantitative Western blotting. Phosphorylation of PKC at S660 (pPKC-S660) was detected with a polyclonal anti-pan PKC phosphorylation state-specific antibody and an [^{125}I]-anti-rabbit antibody. **A.** Time-course of PKC-S660 phosphorylation. **B.** Quantitation of the upper (left panel) and lower (right panel) pPKC-S660 bands in 'A.'. The data from each muscimol-treated group were normalised to the respective untreated control. The results of several experiments (mean ± sem; n = 5-8) were compared with the untreated control using the Student's paired *t* test (* denotes $p<0.05$).

band: 109.7±6.5; $p>0.05$; n=7), 30 min (upper band: 107.2±8.6; $p>0.05$; n=5) (lower band: 108.1±3.8; $p>0.05$; n=6) or 60 min (upper band: 104.4±8.7; $p>0.05$; n=8) (lower band: 114.8±7.3; $p>0.05$; n=8) (Fig. 4.9B). These data demonstrated a small increase in phosphorylation of PKC at S660 in cultured cortical neurons following incubation with muscimol for 15 min, but not 5, 30 or 60 min. This suggested that short-term activation of $GABA_A$ receptors might trigger a small increase in PKC activity in cultured immature cortical neurons.

4.2.5 CaMKII-Dependent Phosphorylation of $GABA_A$ Receptor β3 Subunits *In Situ* in Cultured Immature Cortical Neurons

I next performed pre-labelling experiments with [^{33}P]-orthophosphate (Section 2.5.12), to determine whether muscimol-induced activation of CaMKIIα in cultured immature cortical neurons correlated with a change in the phosphorylation state of $GABA_A$ receptors. Primary cultures of cortical neurons (E17; 6DIV) were incubated at 37 °C in phosphate-free medium for 2 h, and then for a further 4 h in the presence of [^{33}P]-orthophosphate. The neurons were then incubated for a further 5 min in the absence or presence of muscimol (50 μM), with or without KN93 (4 μM; added 20 min prior to treatment) (in HBS). Following the treatments, the neurons were lysed under denaturing conditions for optimal preservation of the phosphorylation state of the receptor subunits. The proteins were then partially renatured and immunoprecipitations were performed using polyclonal anti-β3 or anti-γ2 subunit antibodies, or non-specific rabbit IgG as a control, (Table 2.6) and protein-A beads. Notably, the expression of the γ2 and β3 subunits in cultured immature cortical neurons has previously been confirmed by metabolic labelling with [^{35}S]-methionine and immunoprecipitation using the same antibodies and conditions as in this study (Jovanovic *et al.*, 2004; see also Macdonald and Olsen, 1994). Following the immunoprecipitations, proteins were resolved by SDS-PAGE and [^{33}P]-labelled subunits were visualised by phosphorimaging.

As shown in Figure 4.10, I detected a [^{33}P]-protein band at the predicted molecular weight of the $GABA_A$ receptor β3 subunit, 58 kDa, from immunoprecipitations performed with the anti-β3 subunit antibody. The [^{33}P]-protein band from neurons

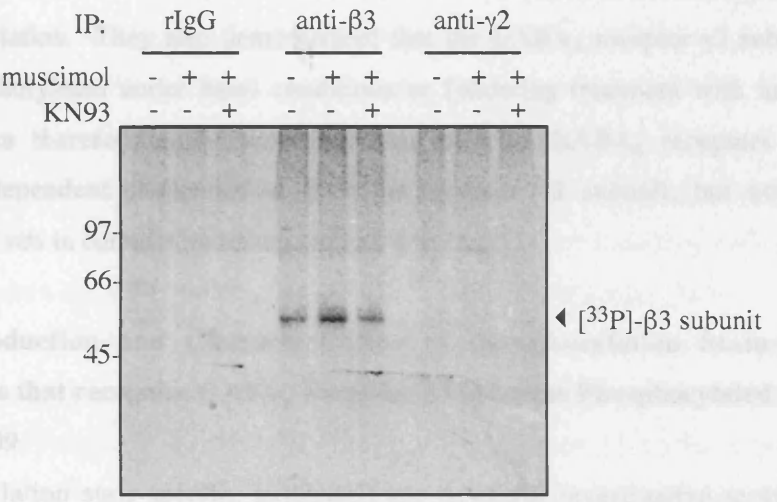


Figure 4.10. CaMKII-dependent phosphorylation of $GABA_A$ receptor β3 subunits *in situ* in immature cortical neurons. Primary cultures of cortical neurons (E17; 6DIV) were pre-labelled with [^{33}P]-orthophosphate in phosphate-free medium for 4 h at 37 °C before being treated for 5 min in the absence (-) or presence (+) of muscimol (50 μM), with (+) or without (-) KN93 (4 μM; added 20 min prior to treatment). Following lysis of neurons, receptor subunits were immunoprecipitated with polyclonal anti-β3 or anti-γ2 subunit antibodies, or non-specific rabbit IgG (rIgG) as a control, and protein-A beads. Proteins were then resolved by SDS-PAGE and [^{33}P]-labelled $GABA_A$ receptor subunits were visualised by autoradiography. 'IP', immunoprecipitation.

treated with muscimol was of greater intensity than that detected under basal conditions, and this enhancement was prevented by co-application of KN93 (Fig. 4.10). I did not detect any [^{33}P]-protein bands from immunoprecipitations performed with the anti-γ2 subunit antibody or non-specific control IgG. These results demonstrated that the $GABA_A$ receptor β3 subunit was phosphorylated under basal conditions, and that muscimol induced a KN93-sensitive increase in β3 subunit phosphorylation. They also demonstrated that the $GABA_A$ receptor γ2 subunit was not phosphorylated under basal conditions or following treatment with muscimol. These data therefore suggested that stimulation of $GABA_A$ receptors induces CaMKII-dependent phosphorylation of the receptor β3 subunit, but not the γ2 subunit, *in situ* in cultured immature cortical neurons.

4.2.6 Production and Characterisation of Phosphorylation State-Specific Antibodies that recognise $GABA_A$ Receptor β3 Subunits Phosphorylated at S383, and at S409

Phosphorylation state-specific antibodies are powerful investigative tools for the detection and quantitation of changes in the phosphorylation state of specific sites of particular proteins. Previous studies using phosphorylation state-specific antibodies have begun to delineate neuronal signalling pathways that lead to changes in the phosphorylation state of specific residues of $GABA_A$ receptor subunits. For example, studies using a phosphorylation state-specific antibody that recognises the $GABA_A$ receptor β3 subunit phosphorylated at S408 and S409 (anti-β3-pS408/409) (Jovanovic *et al.*, 2004) have shown that activation of muscarinic acetylcholine receptors (Brandon *et al.*, 2002), or TrkB neurotrophin receptors (Jovanovic *et al.*, 2004), leads to PKC-dependent phosphorylation of the $GABA_A$ receptor β3 subunit at S408 and S409 in primary cultures of cortical neurons. Furthermore, activation of D_1 dopamine receptors has been shown to induce PKA-dependent phosphorylation of the $GABA_A$ receptor β3 subunit at S408 and S409 in hippocampal slices (Terunuma *et al.*, 2004). I therefore decided to raise phosphorylation state-specific antibodies that recognise the $GABA_A$ receptor β3 subunit phosphorylated at S383, or at S409, to determine whether CaMKII-dependent phosphorylation of the β3 subunit (Fig. 4.10)

occurs at either or both of these residues (the *in vitro* phosphorylation sites) in cultured immature cortical neurons.

4.2.6.1 Design and Production of Peptide Immunogens, and Inoculation of Rabbits

I used short synthetic phospho-peptide immunogens (β3-S383-P or β3-S409-P peptides) for the production of phosphorylation state-specific antibodies that recognise the $GABA_A$ receptor β3 subunit phosphorylated at S383 (anti-β3-pS383 antibody), or at S409 (anti-β3-pS409 antibody) (Section 2.4.3; Tables 2.4 and 2.6). Synthetic peptides were also used to characterise the antibodies (Table 2.4). The synthetic peptides corresponded to a short region (12 amino acids) surrounding either S383 or S409 of the $GABA_A$ receptor β3 subunit, and were modified with phospho-serine residues by chemical phosphorylation, where appropriate (Table 2.4; Section 2.4.3). The peptides also contained an N-terminal cysteine residue for conjugation to the *Limulus* hemocyanin carrier-protein, via the cross-linker sulfo-MBS, where appropriate (Table 2.4; Section 2.4.3). All peptides were synthesised and HPLC purified at The Rockefeller University Protein/DNA Technology Centre in the USA (www.pdtc.rockefeller.edu), and were designed in accordance with the Centre's guidelines (Section 2.4.3). The β3-S383-P and β3-S409-P peptides were each used to raise polyclonal antibodies in two rabbits. This part of the project was contracted out to Cocalico Biologicals Inc, in the USA (Section 2.4.3). Rabbits UCL-103 and UCL-104 were immunised with the β3-S383-P peptide, and rabbits UCL-105 and UCL-106 were immunised with the β3-S409-P peptide. The rabbits were immunised using the standard company protocol, in Freund's Complete Adjuvant.

4.2.6.2 Antibody Screen I

I monitored production of the desired antibodies in the rabbits by testing samples of serum, which had been taken from each rabbit at various stages of the project. A serum sample was obtained before the initial inoculation (pre-immune serum), after the initial inoculation and the second antigen boost (test-bleed 1), and following the third antigen boost (test-bleed 2). A timetable of the project is shown in Table 2.5. In the first antibody screen, I tested the affinity, site-specificity and phospho-specificity of the serum antibodies by using a dot-blot assay with the immunising and control

synthetic peptides (Section 2.5.9; Figs. 4.11 and 4.12). In addition to detecting the desired antibodies, this screen was an important means of quality control, and was crucial for estimating the optimal time for exsanguination. I tested the immunising phospho-peptides, β3-S383-P and β3-S409-P, and a number of control peptides. These included the dephosphorylated forms of the immunising peptides, β3-S383 and β3-S408/S409, respectively, and a form of the β3-S408/S409 peptide that was phosphorylated at S408, β3-S408-P, or on both S408 and S409, β3-S408/S409-P (Table 2.4). To perform the dot-blot assay, I first prepared a peptide grid (one for each serum sample) by pipetting increasing amounts (0, 5, 25, 50 and 100 ng) of each synthetic peptide in a volume of 1 μl onto a piece of PVDF membrane. The peptides on each grid were then probed with one of the serum samples in a Western blot.

I first examined the serum samples obtained from UCL-103 and UCL-104, which had been immunised with the β3-S383-P peptide. As shown in Figure 4.11, the pre-immune serum from both UCL-103 (Fig. 4.11A) and UCL-104 (Fig. 4.11B) did not display immunoreactivity to any of the peptides. However, both rabbits developed immunoreactivity towards the immunising peptides following the initial inoculation and the first two antigen boosts (Fig. 4.11C and D). UCL-103 (test-bleed 1) detected as little as 5 ng of the immunising β3-S383-P peptide (Fig. 4.11C). Immunoreactive spots were also detected with the control β3-S383 peptide, although they were much fainter than those for β3-S383-P (Fig. 4.11C). Immunoreactive spots were not detected in the absence of the peptides, or for any of the other control peptides (Fig. 4.11C). Like UCL-103 (test-bleed 1), UCL-104 (test-bleed 1) detected as little as 5 ng of the immunising β3-S383-P peptide and the control β3-S383 peptide (Fig. 4.11D). Once again, weaker spots were obtained for β3-S383 compared to β3-S383-P (Fig. 4.11D). Although immunoreactive spots were not detected in the absence of the peptides, or for the β3-S408/S409 or β3-S408-P peptides, faint spots were obtained with 25+ ng of the β3-S409-P and β3-S408/S409-P control peptides (Fig. 4.11D). After the third antigen boost, UCL-103 (test-bleed 2) still detected as little as 5 ng of β3-S383-P, and faint bands were observed with only 50 and 100 ng of β3-S383 (Fig. 4.11E). Very faint dots were also detected with 25+ ng of β3-S409-P, and 50+ ng of β3-S408/S409-P (Fig. 4.11E). Immunoreactive spots were not detected in

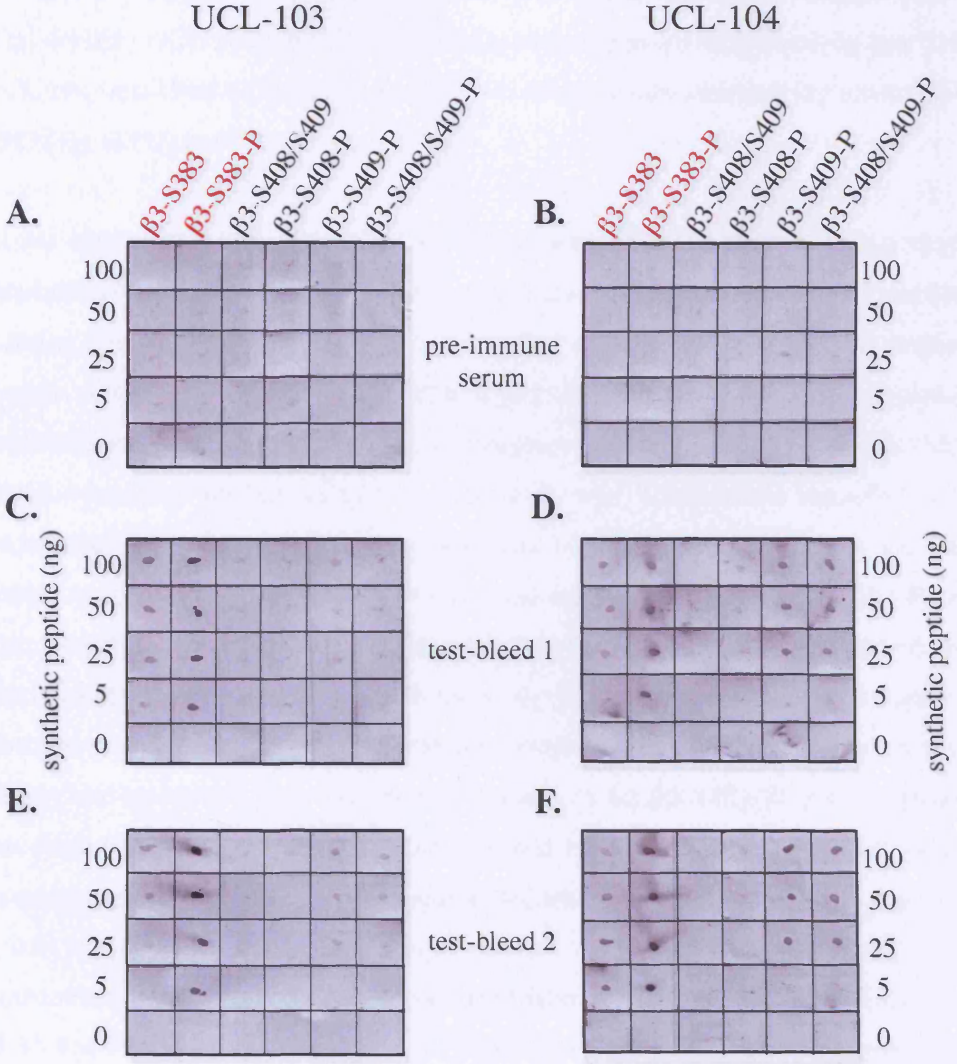


Figure 4.11. Immunoreactivity of UCL-103 and UCL-104 to the synthetic immunising and control peptides. Dot-blot assays were performed using crude serum samples obtained from two rabbits (UCL-103 and UCL-104) at different stages of antibody production, and the synthetic immunising (β3-S383-P) and control (β3-S383, β3-S408/S409, β3-S408-P, β3-S409-P and β3-S408/S409-P) peptides. 'P' indicates 'phospho'. Increasing amounts (0, 5, 25, 50 and 100 ng) of each peptide were spotted onto PVDF membranes and Western blots were perfomed using a 1:100 dilution of the crude serum samples obtained from UCL-103 (**A.**, **C.** and **E.**) and UCL-104 (**B.**, **D.** and **F.**), before immunisation (**A.** and **B.**), after immunisation and two antigen boosts (**C.** and **D.**), and following a further antigen boost (**E.** and **F.**). A secondary anti-rabbit-AP antibody was used for detection. Key peptides involved in the charaterisation process are highlighted in red.

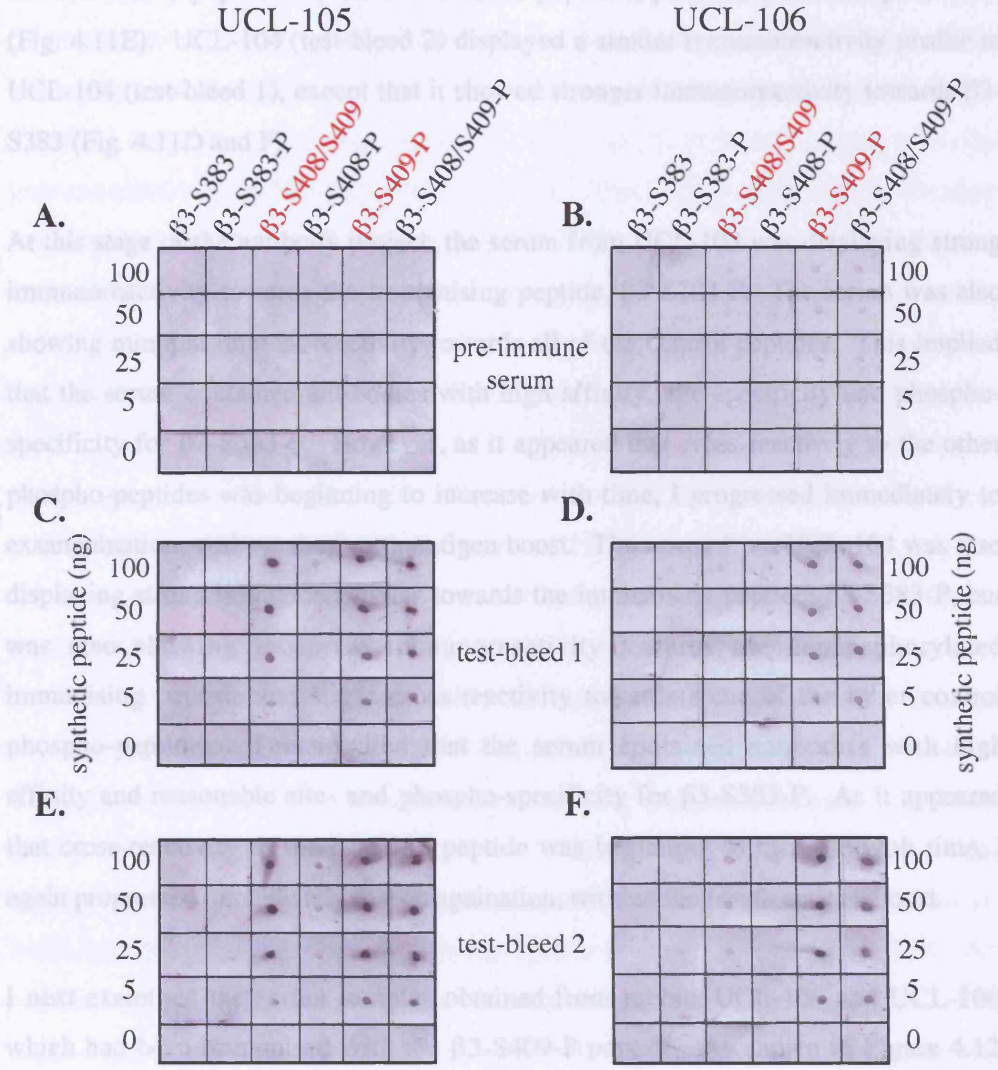


Figure 4.12. Immunoreactivity of UCL-105 and UCL-106 to the synthetic immunising and control peptides. Dot-blot assays were performed using crude serum samples obtained from two rabbits (UCL-105 and UCL-106) at different stages of antibody production, and the synthetic immunising (β3-S409-P) and control (β3-S383, β3-S383-P, β3-S408/S409, β3-S408-P and β3-S408/S409-P) peptides. 'P' indicates 'phospho'. Increasing amounts (0, 5, 25, 50 and 100 ng) of each peptide were spotted onto PVDF membranes and Western blots were perfomed using a 1:100 dilution of the crude serum samples obtained from UCL-105 (**A.**, **C.** and **E.**) and UCL-106 (**B.**, **D.** and **F.**), before immunisation (**A.** and **B.**), after immunisation and two antigen boosts (**C.** and **D.**), and following a further antigen boost (**E.** and **F.**). A secondary anti-rabbit-AP antibody was used for detection. Key peptides involved in the charaterisation process are highlighted in red.

the absence of peptides or with the control peptides, β3-S408/S409 and β3-S408-P (Fig. 4.11E). UCL-104 (test-bleed 2) displayed a similar immunoreactivity profile to UCL-104 (test-bleed 1), except that it showed stronger immunoreactivity towards β3-S383 (Fig. 4.11D and F).

At this stage of the antibody project, the serum from UCL-103 was displaying strong immunoreactivity towards the immunising peptide, β3-S383-P. The serum was also showing minimal immunoreactivity towards all of the control peptides. This implied that the serum contained antibodies with high affinity, site-specificity and phospho-specificity for β3-S383-P. However, as it appeared that cross-reactivity to the other phospho-peptides was beginning to increase with time, I progressed immediately to exsanguination, without the fourth antigen boost. The serum from UCL-104 was also displaying strong immunoreactivity towards the immunising peptide, β3-S383-P, but was also showing moderate immunoreactivity towards the dephosphorylated immunising peptide and slight cross-reactivity towards some of the other control phospho-peptides. This implied that the serum contained antibodies with high affinity and reasonable site- and phospho-specificity for β3-S383-P. As it appeared that cross-reactivity to the β3-S383 peptide was beginning to increase with time, I again progressed immediately to exsanguination, without the fourth antigen boost.

I next examined the serum samples obtained from rabbits UCL-105 and UCL-106, which had been immunised with the β3-S409-P peptide. As shown in Figure 4.12, the pre-immune serum from both UCL-105 (Fig. 4.12A) and UCL-106 (Fig. 4.12B) did not display immunoreactivity towards any of the peptides. However, both rabbits had developed immunoreactivity towards the immunising peptides following the initial inoculation and the first two antigen boosts (Fig. 4.12C and D). UCL-105 (test-bleed 1) detected as little as 5 ng of the immunising β3-S409-P peptide and the β3-S408/S409-P peptide (Fig. 4.12C). Cross-reactivity was also detected with the β3-S408/S409 peptides, and weakly with 100 ng of β3-S408-P (Fig. 4.12C). UCL-105 (test-bleed 1) did not display any immunoreactivity in the absence of peptide or towards the remaining control peptides, β3-S383 and β3-S383-P (Fig. 4.12C). Like UCL-105 (test-bleed 1), UCL-106 (test-bleed 1) detected as little as 5 ng of the

immunising β3-S409-P peptide and the β3-S408/S409-P peptide (Fig. 4.12D). Immunoreactive spots were not detected in the absence of peptide, or with any of the remaining control peptides, β3-S383, β3-S383-P, β3-S408/S409 and β3-S408-P (Fig. 4.12D). After the third antigen boost, UCL-105 (test-bleed 2) had a similar immunoreactivity profile as UCL-105 (test-bleed 1), except for a stronger immunoreactivity towards 25+ ng of β3-S408-P (Fig. 4.12C and E). UCL-106 (test-bleed 2) also had a similar immunoreactivity profile to UCL-106 (test-bleed 1), except for weak immunoreactivity towards 25+ ng of β3-S408/S409, and stronger immunoreactivity towards β3-S409-P than β3-S408/S409-P (Fig. 4.12D and F).

At this stage of the antibody project, the serum from UCL-105 was displaying strong immunoreactivity towards the immunising peptide, β3-S409-P. The serum was also showing moderate immunoreactivity towards the dephosphorylated β3-S408/S409 peptide and the di-phosphorylated β3-S408/S409-P peptide. This demonstrated that the UCL-105 serum contained antibodies with high affinity but only slight site- and phospho-specificity for β3-S409-P. As it appeared that cross-reactivity to β3-S408-P was increasing with time, I progressed immediately to exsanguination, without the fourth antigen boost. The serum from UCL-106 was also displaying strong immunoreactivity towards the immunising peptide, β3-S409-P, but was also showing weak immunoreactivity towards 25+ ng of the dephosphorylated β3-S408/S409 peptide and modest cross-reactivity towards the β3-S408/S409-P peptide. This implied that the serum contained antibodies with high affinity and reasonable site- and phospho-specificity for β3-S409-P. Although the cross-reactivity towards β3-S408/S409-P had been reduced over time, it appeared that cross-reactivity to the dephosphorylated β3-S408/S409 peptide was beginning to increase with time. I therefore progressed immediately to exsanguination, without the fourth antigen boost.

4.2.6.3 Antibody Screen II

In the second antibody screen, I tested the affinity, site-specificity and phospho-specificity of the affinity-purified polyclonal anti-β3-pS383 and anti-β3-pS409 antibodies for purified, bacterially expressed GST-β3 fusion proteins that had been phosphorylated, *in vitro*, by purified CaMKII. These included wild type GST-β3, and

mutant GST-β3 fusion proteins in which various serine residues were replaced with alanine residues, namely GST-β3-S383A, GST-β3-S408A, GST-β3-S409A, GST-β3-S383A/S409A and GST-β3-S408A/S409A (Table 2.2). I chose the exsanguination sera from UCL-103 and UCL-106 as the preferred sera for affinity-purification, based on the findings of the first antibody screen (Section 4.2.6.2). Anti-β3-pS383 and anti-β3-pS409 antibodies were affinity-purified from the respective sera using protein-A columns, and β3-S383-P or β3-S409-P peptide columns (Section 2.5.2; Table 2.6).

I performed *in vitro* kinase assays essentially as described in Section 4.2.1, except that two sets of reactions (one 'hot' and one 'cold') were run in parallel (Section 2.5.13). The kinase reactions were set up in CaMKII Buffer, with 10 μg each of the wild type or mutant GST-β3 fusion proteins, purified CaMKII (a generous gift of Paul Greengard) and calmodulin. Before addition of the ATP, 10% (v/v) of each assay mix was transferred to a new tube to form a duplicate second set of assays. The first set of reactions commenced upon addition of ATP only ('cold' reactions), and the second set of assays upon addition of ATP and [γ-^{32}P]-ATP ('hot' reactions). Both sets of reactions were incubated in parallel at 30 °C for 15 min. Following SDS-PAGE, [^{32}P]-labelled proteins from the second 'hot' set of reactions were detected by phosphorimaging. For the first 'cold' set, 1 μg of the respective GST-fusion protein from each of the assays was resolved by SDS-PAGE (one set of GST-fusion proteins per antibody) and analysed by Western blotting with the affinity-purified anti-β3-pS383 or anti-β3-pS409 antibodies.

As shown in Figure 4.13A, [^{32}P]-labelled protein bands were detected at the predicted molecular weight of the wild type and mutant GST-β3-fusion proteins (~46 kDa) from kinase assays performed with GST-β3, GST-β3-S383A, GST-β3-S408A, GST-β3-S409A, and GST-β3-S408A/S409A. [^{32}P]-protein bands were not obtained with GST-β3 in the absence of kinase (the mock reaction), with GST-β3-S383A/S409A (in which both phosphorylation sites were mutated) or with GST alone (Fig. 4.13A). This demonstrated, in addition to the previous findings in Section 4.2.1, that CaMKII phosphorylated the GST-β3 fusion proteins at S383 and S409. Furthermore, I was confident that the pattern of phosphorylation that was demonstrated in the 'hot'

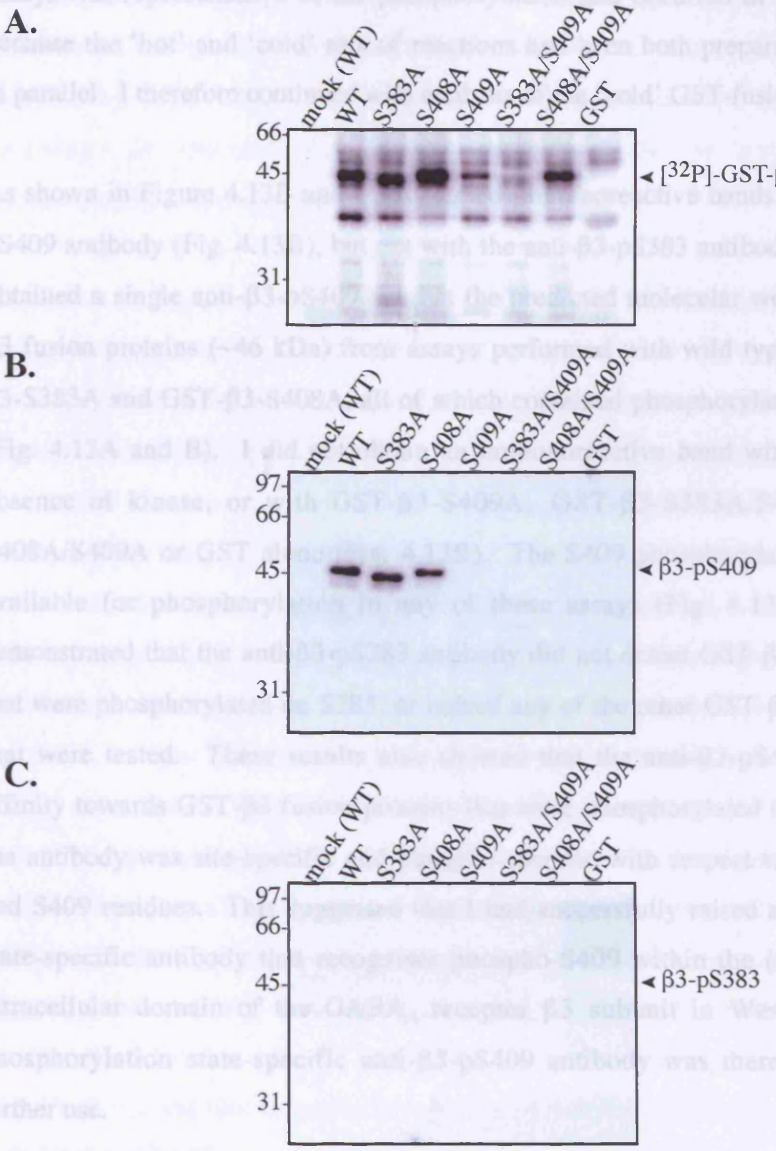


Figure 4.13. The anti-β3-pS409 antibody specifically recognises GST-β3 phosphorylated at S409. *In vitro* kinase assays were performed in the presence of purified CaMKII and 10 μg each of wild type (WT) GST-β3, GST-β3-S383A, GST-β3-S408A, GST-β3-S409A, GST-β3-S383A/S409A, GST-β3-S408A/S409A and GST, or in the absence of kinase (mock; with GST-β3) for 15 min at 30 °C. Before addition of the ATP mix, the reaction mixes were divided into two. **A.** The first set of assays were performed in the presence of ATP and [γ-^{32}P]-ATP. After resolving the proteins by SDS-PAGE, [^{32}P]-GST-fusion proteins were detected by phosphorimaging. **B. and C.** The second set of assays were performed in parallel to those in 'A.' in the presence of ATP. After resolving a fraction of the assay (1 μg of each GST-fusion protein) by SDS-PAGE, the proteins were probed with the affinity-purified anti-β3-pS409 antibody (B.) or anti-β3-pS383 antibody (C.), and an [^{125}I]-anti-rabbit antibody.

assays was representative of the phosphorylation that occurred in the 'cold' assays, because the 'hot' and 'cold' sets of reactions had been both prepared and performed in parallel. I therefore continued with analysis of the 'cold' GST-fusion proteins.

As shown in Figure 4.13B and C, I detected immunoreactive bands with the anti-β3-pS409 antibody (Fig. 4.13B), but not with the anti-β3-pS383 antibody (Fig. 4.13C). I obtained a single anti-β3-pS409 band at the predicted molecular weight of the GST-β3 fusion proteins (~46 kDa) from assays performed with wild type GST-β3, GST-β3-S383A and GST-β3-S408A, all of which contained phosphorylated S409 residues (Fig. 4.13A and B). I did not obtain an immunoreactive band with GST-β3 in the absence of kinase, or with GST-β3-S409A, GST-β3-S383A/S409A, GST-β3-S408A/S409A or GST alone (Fig. 4.13B). The S409 phosphorylation site was not available for phosphorylation in any of these assays (Fig. 4.13A). These data demonstrated that the anti-β3-pS383 antibody did not detect GST-β3 fusion proteins that were phosphorylated on S383, or indeed any of the other GST-β3 fusion proteins that were tested. These results also showed that the anti-β3-pS409 antibody had affinity towards GST-β3 fusion proteins that were phosphorylated on S409, and that the antibody was site-specific and phospho-specific with respect to the S383, S408 and S409 residues. This suggested that I had successfully raised a phosphorylation state-specific antibody that recognises phospho-S409 within the (denatured) major intracellular domain of the $GABA_A$ receptor β3 subunit in Western blots. The phosphorylation state-specific anti-β3-pS409 antibody was therefore suitable for further use.

4.2.7 Analysis of $GABA_A$ Receptor Phosphorylation in Cultured Immature Cortical Neurons using Phosphorylation State-Specific Antibodies

I have previously demonstrated agonist-induced KN93-sensitive phosphorylation of $GABA_A$ receptor β3 subunits in cultured immature cortical neurons (Fig. 4.10). To examine this further, I used phosphorylation state-specific antibodies to analyse the phosphorylation of specific sites within the β3 subunits following agonist treatment. Primary cultures of cortical neurons (E17; 6DIV) were incubated for 5 min at 37 °C in the absence or presence of muscimol (50 μM), with or without KN93 (4 μM; added

20 min prior to treatment) (in HBS). Following the treatments, the neurons were lysed under denaturing conditions. Equal amounts of protein (250 μg) were then resolved by SDS-PAGE, and probed in a Western blot with an anti-β3 subunit antibody, or the affinity-purified anti-β3-pS409 or anti-β3-pS408/S409 phosphorylation state-specific antibodies (Table 2.6).

As shown in Figure 4.14, an immunoreactive band was detected at the predicted molecular weight of the β3 subunit (58 kDa) from control and treated neurons with the anti-β3 subunit antibody and the anti-β3-pS408/S409 antibody. The band obtained with the anti-β3-pS408/S409 antibody was stronger in the muscimol-treated lane than in the untreated control lane, and this signal enhancement appeared to be at least partially blocked by KN93 (Fig. 4.14). However, I did not detect a 58-kDa immunoreactive band from control or treated neurons with the anti-β3-pS409 antibody (Fig. 4.14). These results demonstrated that in cultured immature cortical neurons, the $GABA_A$ receptor β3 subunit was basally phosphorylated at both S408 and S409, and that this phosphorylation was increased following muscimol treatment. As this enhancement appeared to be at least partially sensitive to KN93, it is possible that it was partly dependent on CaMKII. These data also demonstrated that the $GABA_A$ receptor β3 subunit was not phosphorylated to a detectable level at the single S409 site under basal conditions or following treatment with muscimol. These results suggested that muscimol-induced CaMKII-dependent phosphorylation of the $GABA_A$ receptor β3 subunit in cultured immature cortical neurons (Fig. 4.10) does not occur at S409 alone, and that it instead involves phosphorylation of other residues, such as both S408 and S409.

4.3 Discussion

Protein phosphorylation is a major mechanism for modulating $GABA_A$ receptor function and the efficacy of inhibitory synaptic transmission (Moss and Smart, 2001; Section 1.7). Although CaMKII has been shown to physically interact with $GABA_A$ receptors, and to phosphorylate the major intracellular domains of receptor subunits, *in vitro*, it remains to be determined whether $GABA_A$ receptors are substrates of CaMKII in neurons. In this study, I investigated various aspects of $GABA_A$ receptor

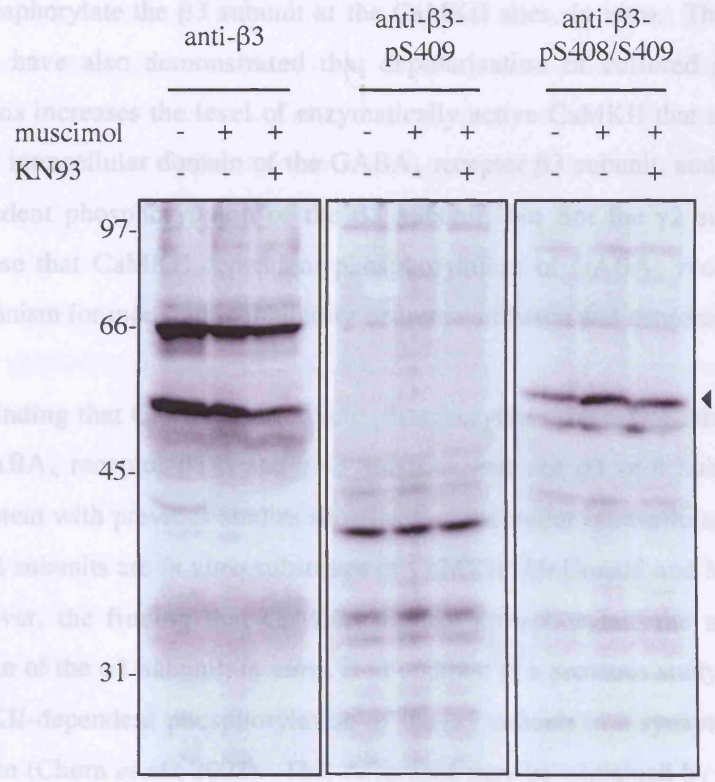


Figure 4.14. Analysis of agonist-induced phosphorylation of the $GABA_A$ receptor β3 subunit in immature cortical neurons. Primary cultures of cortical neurons (E17; 6DIV) were incubated in the absence (-) or presence (+) of muscimol (50 μM) with (+) or without (-) KN93 (4 μM; 20 min pre-incubation) for 5 min at 37 °C. Neurons were lysed under denaturing conditions, and equal amounts of protein (250 μg) were resolved by SDS-PAGE. Proteins were then probed with a polyclonal anti-β3 subunit antibody, or a phosphorylation state-specific antibody that recognises the $GABA_A$ receptor β3 subunit phosphorylated at S409 (anti-β3-pS409), or S408 and S409 (anti-β3-pS408/S409), and an [^{125}I]-anti-rabbit antibody.

phosphorylation by CaMKII, with the aim of further understanding the cellular mechanisms that control the phosphorylation state of these receptors. This study has provided evidence to suggest that CaMKII selectively phosphorylates the $GABA_A$ receptor β and γ subunits, *in vitro*, and that the γ1 and γ3 subunits are novel *in vitro* substrates of CaMKII. Furthermore, that PP1, PP2A and PP2C, but not PP2B, dephosphorylate the β3 subunit at the CaMKII sites, *in vitro*. The results from this study have also demonstrated that depolarisation of cultured immature cortical neurons increases the level of enzymatically active CaMKII that associates with the major intracellular domain of the $GABA_A$ receptor β3 subunit, and triggers CaMKII-dependent phosphorylation of the β3 subunit, but not the γ2 subunit, *in situ*. I propose that CaMKII-dependent phosphorylation of $GABA_A$ receptors is a major mechanism for modulating inhibitory neurotransmission and neuronal excitability.

The finding that CaMKII selectively phosphorylates the major intracellular domains of $GABA_A$ receptor β1-3 and γ1-3 subunits, but not α1 or δ subunits, *in vitro*, is consistent with previous studies showing that the major intracellular domains of the β and γ2 subunits are *in vitro* substrates of CaMKII (McDonald and Moss, 1994, 1997). However, the finding that CaMKII did not phosphorylate the major intracellular domain of the α1 subunit, *in vitro*, is in contrast to a previous study that demonstrates CaMKII-dependent phosphorylation of the α1 subunit in a synaptosomal membrane fraction (Churn *et al.*, 2002). This difference may be explained by the presence of an unidentified $GABA_A$ receptor kinase downstream of CaMKII in the synaptosomal membrane fraction. Alternatively, it is possible that CaMKII is able to phosphorylate the α1 subunit when it is co-assembled with other subunits in a receptor complex in its full-length native state. Further studies are required to investigate whether the other α subunits, α2-6, are substrates of CaMKII, and to identify the sites of phosphorylation in the novel subunit substrates, γ1 and γ3. Nevertheless, these *in vitro* findings suggest that CaMKII holoenzymes may selectively phosphorylate the major intracellular domains of $GABA_A$ receptor β and γ subunits in neurons. This selectivity could provide a potential mechanism for CaMKII to differentially regulate distinct $GABA_A$ receptor populations. Although, as the results of *in vitro* experiments do not always agree with the findings of experiments performed *in situ*, in neurons or

heterologous cells, these results can only be used as a guide to cellular phosphorylation events (Moss *et al.*, 1992a,b; McDonald and Moss, 1997; McDonald *et al.*, 1998; Krishek *et al.*, 1994; Brandon *et al.*, 2000, 2003; Section 1.7.2; Table 1.1). In Chapter 3, I found that native CaMKII bound to the major intracellular domains of $GABA_A$ receptor α1, β1-3, γ1-3 and δ subunits. This suggests that the selectivity of subunit phosphorylation observed *in vitro* is due to the recognition of particular consensus phosphorylation motifs, rather than the differential binding of CaMKII. However, in intact cells, binding may be a prerequisite of phosphorylation, and may control accessibility of the kinase to the phosphorylation sites.

My results confirmed previous studies showing that CaMKII phosphorylates the major intracellular domain of the β3 subunit at S383 (RKQSMPK) and S409 (RRRSSQLK) (McDonald and Moss, 1997). The sequences preceding each of these sites conform with the consensus phosphorylation motif for CaMKII, Rxx(S/T) (where x is equivalent to any amino acid) (Kennelly and Krebs, 1991). However, consensus motifs can only predict the actual sites of protein phosphorylation. For example, I (this study), and others (McDonald and Moss, 1997), did not detect phosphorylation of the β3 subunit at S408, which forms part of a consensus CaMKII phosphorylation motif. Similarly, the identification of these *in vitro* phosphorylation sites can only serve as a guide to the sites that may be phosphorylated by CaMKII in neurons. S383 is conserved in the β1 and β3 subunits, but not the β2 subunit. In contrast, S409 is conserved in all three β subunits. If both of these residues are CaMKII substrates in neurons, then the different patterns of subunit conservation may provide a mechanism for CaMKII to differentially phosphorylate (and regulate) distinct populations of $GABA_A$ receptors.

My analysis of the phosphorylation of GST-β3 by purified CaMKII revealed a maximum stoichiometry of phosphorylation of ~0.22 mol. phosphate mol. protein^{-1}. This compared well with the value previously obtained (0.4 mol. phosphate mol. protein^{-1}), which could not be enhanced by increasing enzyme concentration or assay duration (McDonald and Moss, 1997). The knowledge that these assay conditions supported a good stoichiometry of phosphorylation provided a control for the

preparative phosphorylation of the GST-β3 fusion proteins for the phosphatase assays.

The finding that PP1, PP2A and PP2C, but not PP2B, each dephosphorylate the major intracellular domain of the $GABA_A$ receptor β3 subunit at S383 and S409, *in vitro*, has important implications for the dephosphorylation of $GABA_A$ receptors *in vivo*. However, as with the *in vitro* kinase assays, further studies are required to determine whether these findings are biologically significant in cells. Nevertheless, my findings are consistent with previous studies showing dephosphorylation of the major intracellular domain of the $GABA_A$ receptor β3 subunit, which has been phosphorylated by PKA at S409 (McDonald and Moss, 1997), by PP1 and PP2A, but not PP2B (Terunuma *et al.*, 2004). Previous studies have also demonstrated that PP1, PP2A and PP2B associate with neuronal $GABA_A$ receptor complexes (Terunuma *et al.*, 2004; Jovanovic *et al.*, 2004; Wang *et al.*, 2003a; Section 1.7.2.2). It will therefore be of interest to confirm my findings *in situ* using phosphorylation state-specific antibodies or pre-labelling assays, and activators/inhibitors of the individual phosphatases. The investigation of PP2B will be of particular interest, because like CaMKII, this enzyme is activated by intracellular changes in the levels of calcium and CaM (Cohen, 1989).

The results of the 'pull-down'-kinase experiments suggested that depolarisation of cultured immature cortical neurons by KCl triggers an increase in the level of enzymatically active CaMKII that binds to GST-β3. Previous studies have shown that KCl triggers depolarisation of cultured immature neurons, calcium influx and phosphorylation of CaMKII (Borodinsky *et al.*, 2002, 2003). In Chapter 3 of this thesis, I found that only a constitutively active form of recombinant CaMKIIα, CaMKIIα-T286D, which mimics T286-autophosphorylation, bound to GST-β3 to a detectable level (Section 3.2.3). It is therefore possible that the endogenous T286-phosphorylated CaMKII also has a much greater binding affinity for GST-β3 than the non-T286-phosphorylated CaMKII. If so, then increasing the neuronal pool of the phosphorylated kinase (by KCl treatment) could enhance the level of enzymatically active CaMKII (and the total level of CaMKII) that binds to GST-β3. However, I

cannot exclude the possibility that the endogenous T286-phosphorylated and non-T286-phosphorylated forms of CaMKII have equivalent binding affinity for GST-β3, and that increasing the neuronal pool of phosphorylated CaMKIIα-T286 simply increases the probability of the phosphorylated, rather than the non-phosphorylated kinase from binding to GST-β3 (and that the total level of CaMKII that binds remains unchanged). Nevertheless, these results suggest that depolarisation of neurons and stimulation of CaMKIIα-T286 autophosphorylation could enhance $GABA_A$ receptor-associated CaMKII activity. However, further experiments are required to determine whether the targeting of CaMKII to $GABA_A$ receptors in neurons requires autophosphorylation.

It is important to note that a previous study has shown that CaMKII does not bind and phosphorylate GST-β1 (Brandon *et al.*, 1999). Like GST-β3, GST-β1 is an *in vitro* substrate of purified CaMKII (McDonald and Moss, 1994), and a binding partner of CaMKII from brain (Section 3.2.1). It is perhaps surprising therefore, that CaMKII binds and phosphorylates GST-β3, but not GST-β1. A possible explanation for this apparent discrepancy is that CaMKII does bind and phosphorylate GST-β1 but that this effect was not detected in the previous study (Brandon *et al.*, 1999). This is because the lack of associated CaMKII activity was inferred from the finding that the CaMKII inhibitor, W7, did not reduce GST-β1-associated kinase activity (Brandon *et al.*, 1999). Notably, W7 is a CaM antagonist (Roufogalis *et al.*, 1983). As W7 was added to the *in vitro* kinase assay after binding had occurred, and as CaMKII gains autonomous activity following T286-phosphorylation and removal of calcium and CaM (Section 1.8), then any CaMKII that bound to GST-β1 in the T286-phosphorylated state would be unlikely to be inhibited by W7. Given the finding that recombinant CaMKII only binds to receptor β subunits in the T286-phosphorylated state, this suggests that W7 may not cause a noticeable reduction in GST-β1-associated CaMKII activity. In my assays, I avoided this problem by pre-treating live cultured neurons with the CaMKII inhibitor, KN93 (which also competes with calmodulin), so that when the neurons were subsequently depolarised, any T286-autophosphorylation and hence CaMKII activation would be prevented or reduced. Although KN93 prevented a significant KCl-induced increase in GST-β3-associated

kinase activity, it is important to note that this inhibitor did not completely block the effect of KCl (the phosphorylation in assays using neurons co-treated with KCl and KN93 was not significantly different to those treated with KCl alone). It is possible that increasing the n numbers would reveal a significant difference. However, KCl-induced depolarisation may be enhancing the affinity of an additional kinase for GST-β3 (or decreasing the affinity of a phosphatase), which is insensitive to KN93. Hence, blocking CaMKII activation would only partially inhibit the KCl-induced increase in phosphorylation of GST-β3.

My studies demonstrated that in cultured immature cortical neurons, basal phosphorylation of CaMKII at T286 is reduced by BAPTA-AM and KN93, and therefore sensitive to changes in the concentration of calcium and CaM, and the ability of CaMKII to autophosphorylate. However, the results also showed that the total level of CaMKIIα protein is reduced following treatment with BAPTA-AM. This decrease may have caused, or contributed to the reduction in T286-phosphorylation of CaMKII that was detected following BAPTA-AM treatment. It is possible that with higher n numbers, the effect of BAPTA-AM on total levels of CaMKIIα will no longer be significant. However, it is also possible that calcium is required to maintain cellular levels of CaMKIIα protein. Indeed, it is tempting to speculate that this potential calcium-dependent process involves dendritic protein synthesis, as CaMKIIα mRNA has been localised to dendrites (Burgin *et al.*, 1990; Paradies and Steward, 1997), and as local translation of CaMKIIα mRNA has been shown to be essential for maintaining the total level of CaMKIIα protein in neurons, and at PSDs (Miller *et al.*, 2002).

Further investigations in cultured immature cortical neurons, revealed that stimulation of $GABA_A$ receptors leads to an increase in CaMKIIα phosphorylation at T286, which is blocked by KN93 and BAPTA-AM. As muscimol did not alter the total level of CaMKIIα protein, this suggested that activation of $GABA_A$ receptors triggers calcium/CaM-dependent autophosphorylation of CaMKIIα at T286. This is consistent with the finding that activation of $GABA_A$ receptors induces depolarisation and an increase in intracellular calcium concentration in cultured immature cortical

neurons under similar experimental conditions (J.N. Jovanovic, unpublished data). It is also consistent with studies in cultured immature cerebellar granule cells showing that activation of $GABA_A$ receptors triggers an increase in intracellular calcium concentration via L-type voltage-gated calcium channels, and an enhancement in phosphorylation of CaMKII (Borodinsky *et al.*, 2003). A $GABA_A$ receptor-mediated rise in intracellular calcium concentration has also been shown to occur in slices of developing neocortex (Yuste and Katz, 1991; Owens *et al.*, 1996). I also found that the muscimol-induced increase in CaMKII phosphorylation lasted for only 15 minutes (even when muscimol was applied for up to 30 and 60 minutes). The duration of this effect may be dependent upon the action of protein phosphatases on CaMKII and/or rapid sustained buffering of calcium. Alternatively, the effect of muscimol on T286-phosphorylation at later time points (30 and 60 minutes) may have been obscured by the phosphorylation of CaMKII at T286 that occurred under control conditions, or limited by $GABA_A$ receptor desensitisation.

I detected a small (10 to 15%) muscimol-induced increase in phosphorylation of PKC at S660 in immature cultured cortical neurons. This suggested that activation of $GABA_A$ receptors triggers a small increase in PKC activity. However, the increase in PKC activity induced by muscimol in this study was much lower than that observed following treatment of cultured cortical neurons with BDNF (Jovanovic *et al.*, 2004). Further investigations will therefore be important in determining whether there is any functional relevance of this apparent increase in activity. It is possible that the level of PKC phosphorylation detected is underestimated. This is because the experiments were performed with a phosphorylation state-specific antibody that recognises multiple PKC isoforms (α, βI, βII, δ, ε, η and θ), only some of which (α and β) are calcium-dependent (Tanaka and Nishizuka, 1994; Song and Messing, 2005). It would therefore be desirable to conduct further experiments utilising antibodies that recognise specific PKC isoforms phosphorylated at S660. The accuracy of the results from these assays, as well as from those investigating basal and muscimol-induced phosphorylation of CaMKII at T286, could perhaps be improved by generating data that enables the calculation of a ratio of phosphorylated kinase to the total kinase for each treatment. However, the disadvantage of this alternative calculation is that

changes in either phosphorylation or total protein levels would not be apparent. Further experiments could also be performed to increase the statistical confidence of the results, as only small changes in phosphorylation were detected, which may have been a result of investigating a heterogenous population of cells.

My studies demonstrated muscimol-induced CaMKII-dependent phosphorylation of $GABA_A$ receptor β3 subunits, but not γ2 subunits, *in situ* in cultured immature cortical neurons. This is consistent with a number of findings in this thesis. Firstly, that CaMKII forms a native complex with $GABA_A$ receptor β3 subunits in brain. Secondly, that KCl-induced depolarisation of immature cortical neurons increases the level of enzymatically active CaMKII that binds to the major intracellular domain of the β3 subunit, and finally, that treatment of immature cortical neurons with muscimol triggers autophosphorylation of CaMKII at T286. My results are also consistent with a previous study showing that the $GABA_A$ receptor β3 subunit is phosphorylated *in situ* in cultured cortical neurons under basal conditions (Brandon *et al.*, 2000; Jovanovic *et al.*, 2004). The finding that CaMKII does not contribute to this basal phosphorylation (Brandon *et al.*, 2000) further supports the hypothesis that CaMKII-dependent phosphorylation of $GABA_A$ receptors relies upon autophosphorylation-dependent targeting of the kinase to receptor complexes. Although I detected basal phosphorylation of CaMKII at T286 in these primary cultures, this measurement represented the total neuronal pool of CaMKII, and so the level of activated CaMKII at cell-surface $GABA_A$ receptors under basal conditions may still be undetectable or low. These data collectively support the view that in immature cortical neurons, where GABA is excitatory, CaMKII is a component of a $GABA_A$ receptor feedback loop, in which activation of $GABA_A$ receptors leads to depolarisation and autophosphorylation of CaMKII, binding of activated CaMKII to $GABA_A$ receptors and receptor subunit phosphorylation. Notably, these results do not provide conclusive evidence that CaMKII directly phosphorylates the β3 subunit in neurons. Indeed, CaMKII may be positioned upstream of another, possibly unknown kinase. It is also possible that CaMKII-dependent phosphorylation of $GABA_A$ receptors is not dependent upon activation of a calcium-dependent signalling pathway. Indeed, the binding of agonist may induce a conformational change of the

receptor that modifies the relative binding and activity of various protein kinases and phosphatases, including enzymatically active CaMKII.

In this study, I did not detect either basal or muscimol-induced phosphorylation of the $GABA_A$ receptor γ2 subunit *in situ* in cultured cortical neurons. This is consistent with a previous study showing that the $GABA_A$ receptor γ2 subunit is not phosphorylated *in situ* in cultured cortical neurons under basal conditions or following treatment with BDNF (Jovanovic *et al.*, 2004). As CaMKII has been shown to phosphorylate the γ2 subunit at multiple sites *in vitro* (McDonald and Moss, 1994), the findings in this study may further exemplify how kinase substrates can differ between experiments performed *in vitro* and *in situ*. However, it is possible that CaMKII-dependent phosphorylation of γ2 subunits occurs under specific conditions that involve co-activation of other intracellular signalling pathways. Indeed, phosphorylation of the γ2 subunit on tyrosine residues has only been detected in cultured cortical neurons following inhibition of tyrosine phosphatase activity (Brandon *et al.*, 2001). Further studies investigating the phosphorylation of specific γ2 subunit sites in response to activation of distinct intracellular signalling pathways will therefore be of interest.

In this study, I attempted to raise polyclonal phosphorylation state-specific antibodies that recognise $GABA_A$ receptor β3 subunits phosphorylated at S383, and at S409, to further investigate CaMKII-dependent phosphorylation of β3 subunits *in situ* in cultured immature cortical neurons. The anti-β3-pS383 and anti-β3-pS409 antibodies each recognised their respective peptide immunogen with high affinity, site-specificity and phospho-specificity. However, in the second antibody screen, only anti-β3-pS409 was able to recognise phosphorylated GST-β3 fusion proteins, which contain the full-length sequence of the major intracellular domain of the β3 subunit. Anti-β3-pS383 was therefore not suitable for further application. Further attempts to raise such an antibody may involve using a different length and/or sequence of immunising synthetic peptide (Czernik *et al.*, 1997). However, this aspect of the design process is somewhat limited by the amino acid sequence surrounding the phosphorylation site of interest. For example, the immunising peptides for the anti-

β3-pS409 and anti-β3-pS408/409 antibodies contain a greater number of basic amino acids (lysine, arginine and histidine) than the peptide for the anti-β3-pS383 antibody (Table 2.4), residues that are believed to increase the antigenicity of a synthetic peptide (Czernik *et al.*, 1997). Further attempts could also involve coupling the peptide to the carrier protein by introducing the cysteine residue at the C-terminus rather than the N-terminus of the peptide. However, increasing the distance of the phosphorylated residue from the free end of the peptide can have an undesirable effect on antigenicity (Czernik *et al.*, 1997). Although immunisation of a greater number of rabbits may increase the likelihood of raising a phosphorylation state-specific antibody, it is worth noting the general difficulty the Moss laboratory has encountered in raising phosphorylation state-specific antibodies that recognise $GABA_A$ receptor subunits that are phosphorylated at a single residue.

I found that the anti-β3-pS409 antibody recognised the full-length major intracellular domain of the $GABA_A$ receptor β3 subunit when it was phosphorylated at S409, and that the antibody exhibited good affinity, site-specificity and phospho-specificity. However, I did not detect a signal with the anti-β3-pS409 antibody when I probed native β3 subunits from cortical neuronal lysates that had been untreated or depolarised with muscimol. This suggested that S409 alone is not phosphorylated under basal conditions or following muscimol-induced depolarisation (within 5 min). This is consistent with the finding that $GABA_A$ receptor β3 subunits are phosphorylated at S408 and S409 in cultured cortical neurons under basal conditions (Brandon *et al.*, 2000), and following muscimol-induced depolarisation (this study), and the results of the first antibody screen, which demonstrated that the anti-β3-pS409 antibody (UCL-106) had much greater affinity for the synthetic immunising peptide phosphorylated at S409, than that phosphorylated at both S408 and S409. Although further studies are required to show such phospho-specificity with the full-length protein, these findings suggest that the presence of a phosphate group at S408 of the native β3 subunit may occlude detection of phosphorylation at S409 by anti-β3-pS409. Hence, a signal will only be detected with the anti-β3-pS409 antibody when S409 is phosphorylated and S408 is not. Another possible explanation for my findings is that other modifications of native β3 subunits prevent the anti-β3-pS409

antibody from recognising a lone phosphorylated S409 residue. I therefore cannot completely exclude the possibility that S409 alone is phosphorylated in cortical neurons under basal conditions and/or following muscimol-induced depolarisation. It is also possible that the affinity of the anti-β3-pS409 antibody for the native β3-pS409 subunit is too low, and/or that there is an insufficient level of β3-pS409 subunits in cortical neurons to be detected by this antibody.

In this study, I demonstrated that muscimol-induced depolarisation of cultured immature cortical neurons leads to enhanced phosphorylation of S408 and S409, but not S409 alone. This finding was based on the results of experiments using a phosphorylation state-specific antibody that recognises $GABA_A$ receptor β3 subunits phosphorylated at S408 and S409, anti-pS408/S409 (Jovanovic *et al.*, 2004). As muscimol-induced depolarisation was also shown to trigger autophosphorylation and activation of CaMKII, it is possible that activation of CaMKII leads to phosphorylation of S408 (which is not an *in vitro* CaMKII site) and S409. As discussed previously, it is known that differences exist between *in vitro* and *in situ* systems. These differences may arise because of altered accessibility of a kinase to the consensus phosphorylation site in the full-length native protein. As shown with PKA (Section 1.7.2.1.2), this may be a result of interactions that occur between $GABA_A$ receptor subunits and a kinase or kinase-associated protein. It is also possible that CaMKII is upstream of another kinase that phosphorylates S408 and S409. However, as muscimol-induced phosphorylation of S408 and S409 does not appear to be abolished by KN93, then some level of phosphorylation may occur following direct activation of an alternative kinase (or inhibition of a phosphatase). Alternatively, an agonist-induced conformational change of the receptor may lead to altered phosphorylation of these sites (as discussed above). However, it is important to note that the anti-pS408/S409 antibody has never been screened against the wild type full-length $GABA_A$ receptor β3 subunit (β3 subunit that has not been subjected to site-directed mutagenesis) that has been phosphorylated solely on S383 or S409. This antibody may therefore be cross-reacting with native β3 subunits that are phosphorylated at either or both of these sites.

The change in S408/S409 phosphorylation that occurs in cortical neurons following muscimol-induced depolarisation may only account for a fraction of the total β3 subunit phosphorylation I observed *in situ*. It appears unlikely that the S409 residue is phosphorylated alone, but it is possible that the S383 residue (the other *in vitro* CaMKII site in the β3 subunit (McDonald and Moss, 1994, 1997)) is a site of CaMKII-dependent activity. Indeed, experiments involving pre-labelling of NG108-15 cells (that have been transfected with CaMKII) with [^{32}P]-orthophosphate have shown that KCl-induced depolarisation leads to phosphorylation of the $GABA_A$ receptor β3 subunit predominantly at S383 (C.M. Houston, unpublished data). Further attempts to raise a phosphorylation state-specific antibody that recognises the β3 subunit phosphorylated at S383 may therefore provide a valuable tool for further investigation of CaMKII-dependent phosphorylation of $GABA_A$ receptors in neurons.

4.4 Conclusions

Overall, I conclude that CaMKII selectively phosphorylates the $GABA_A$ receptor β1–3 and γ1–3 subunits, but not the α1 or δ subunits, *in vitro*. Furthermore, I have found that the β3 subunit is dephosphorylated at the CaMKII sites by PP1, PP2A and PP2C, but not PP2B, *in vitro*. Depolarisation of cultured immature cortical neurons was found to enhance the level of enzymatically active CaMKII that binds to the major intracellular domain of the $GABA_A$ receptor β3 subunit, and to cause CaMKII-dependent phosphorylation of the β3 subunit, but not the γ2 subunit, *in situ*. Experiments using phosphorylation state-specific antibodies further suggest that the β3 subunit is not phosphorylated at S409, and that some phosphorylation occurs at both S408 and S409. CaMKII-dependent phosphorylation of $GABA_A$ receptors may therefore play a major role in regulating the activity of $GABA_A$ receptors in brain, and therefore have significant consequences for the efficacy of inhibitory synaptic transmission and neuronal excitability.

CHAPTER 5

Molecular Determinants of AP2 Binding to $GABA_A$ Receptors

5.1 Background

The membrane stability and functional properties of synaptic $GABA_A$ receptors are critical determinants of the efficacy of central inhibitory synapses. Several studies have demonstrated that synaptic strength is directly related to the number of postsynaptic $GABA_A$ receptors, and that modulation of cellular processes that insert or remove $GABA_A$ receptors into or from the neuronal membrane can control synaptic efficacy. For example, variability in the amplitude of miniature inhibitory postsynaptic currents (mIPSCs) in cerebellar stellate cells has been attributed to differences in the number of postsynaptic $GABA_A$ receptors (Nusser *et al.*, 1997). Following kindling-induced epilepsy, potentiation of postsynaptic responses in granule cells of the dentate gyrus is caused by an increase in the number of synaptic $GABA_A$ receptors (Otis *et al.*, 1994; Nusser *et al.*, 1998b). Similarly, treatment of cultured neurons with insulin leads to a rapid enhancement in the number of postsynaptic $GABA_A$ receptors and the amplitude of mIPSCs (Wan *et al.*, 1997b; Wang *et al.*, 2003b). Conversely, treatment of cultured hippocampal neurons with BDNF triggers a reduction in the amplitude of mIPSCs (Brunig *et al.*, 2001; Jovanovic *et al.*, 2004), and this is accompanied by a decrease in postsynaptic $GABA_A$ receptor immunoreactivity (Brunig *et al.*, 2001). A number of mechanisms may contribute to such changes in the number of postsynaptic $GABA_A$ receptors. These include increased or decreased rates of $GABA_A$ receptor endocytosis, membrane insertion and/or degradation, and an alteration in the distribution of receptors on the cell surface. Understanding the molecular mechanisms that are involved in these processes is therefore fundamental to our understanding of GABAergic neurotransmission and plasticity at inhibitory synapses.

Clathrin-mediated endocytosis is a major mechanism by which many neuronal receptors are internalised from the neuronal surface to intracellular compartments (Chu *et al.*, 1997; Carroll *et al.*, 1999; Man *et al.*, 2000; Pitcher *et al.*, 1998). This process (reviewed in Mousavi *et al.*, 2004) begins with the assembly of a clathrin coat on the cytoplasmic side of the membrane (reviewed in Brodsky *et al.*, 2001). This comprises the coat protein clathrin, a member of the family of clathrin adaptor proteins (Hirst and Robinson, 1998), usually the adaptor-binding protein 2 (AP2), and

several clathrin/endocytic accessory factors, such as amphiphysin and AP180 (Fig. 5.1A; reviewed in Slepnev and De Camilli, 2000 and Traub, 2003). The adaptor proteins selectively bind membrane cargo proteins and lipids, and recruit clathrin and the other coat components to the neuronal surface. All binding is highly co-operative, and leads to formation of a clathrin polyhedral lattice, which induces deep invagination of the membrane and production of a clathrin-coated pit (Brodsky *et al.*, 2001; Slepnev and De Camilli, 2000; Rappoport *et al.*, 2004). The coated pit then undergoes dynamin-dependent scission to generate a free clathrin-coated vesicle (Brodsky *et al.*, 2001). This transient structure is immediately uncoated to enable fusion with an early/sorting endosome (reviewed in Mellman, 1996, and Hirst and Robinson, 1998). At this juncture, the internalised receptors are directed to various cellular destinations: they may be sequestered in endosomes, targeted for degradation or recycled to the plasma membrane, in parallel with delivery of receptors from the *de novo* synthetic pathway (Mellman, 1996).

$GABA_A$ receptors undergo constitutive and agonist-dependent internalisation (Kittler *et al.*, 2000a, 2005; Connolly *et al.*, 1999a,b; Herring *et al.*, 2003, 2005; Calkin and Barnes, 1994; Calkin *et al.*, 1994; Tehrani and Barnes, 1991). Several lines of evidence suggest that $GABA_A$ receptors are removed from the neuron surface to intracellular compartments via a clathrin-mediated, dynamin-dependent endocytic pathway (Kittler *et al.*, 2000a, 2005; see also Connolly *et al.*, 1999b and Herring *et al.*, 2003). For example, a number of studies have shown that $GABA_A$ receptors are co-purified with clathrin-coated vesicles from brain (Tehrani and Barnes, 1993; Tehrani *et al.*, 1997; Kumar *et al.*, 2003; Tehrani and Barnes, 1997). Furthermore, peptide blockade of the interaction between amphiphysin and dynamin leads to inhibition of $GABA_A$ receptor endocytosis in primary cultures of hippocampal neurons. This causes an increase in the amplitude of mIPSCs, which has been attributed to a corresponding increase in the number of postsynaptic $GABA_A$ receptors (Kittler *et al.*, 2000a, 2005).

The AP2 adaptor plays a pivotal role in clathrin-mediated endocytosis. AP2 is a stable heterotetrameric complex that consists of two large (~100 kDa) adaptins, α and

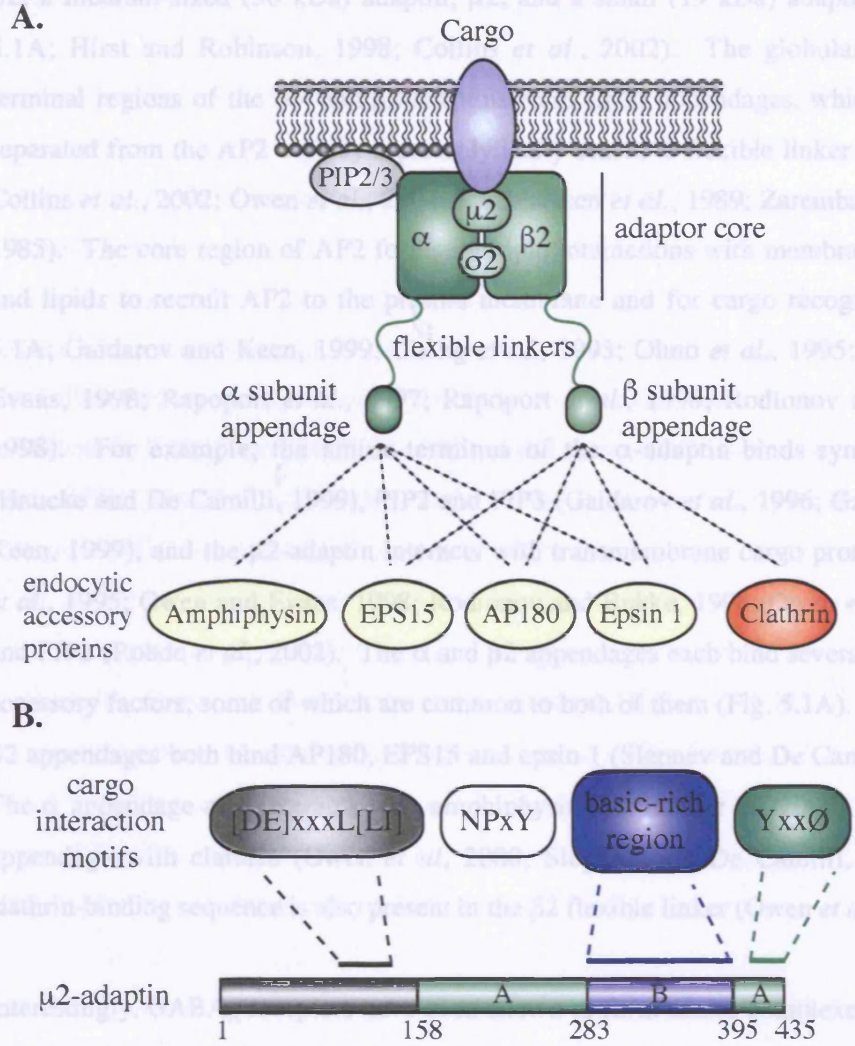


Figure 5.1. Schematic representation of the domain structure of AP2 and the μ2-adaptin. A. Schematic diagram of the heterotetrameric AP2 complex that comprises 2 large (~100 kDa) adaptins, α and β2, 1 medium (50 kDa) adaptin, μ2, and a small (19 kDa) adaptin, σ2. The core region and the flexible linkers to the α and β2 appendages are indicated. The core region mediates multiple interactions with membrane proteins and lipids to anchor AP2 to the membrane and for cargo recognition. Clathrin binds to the appendage and flexible linker of β2-adaptin. The α and β2 appendages each bind several endocytic accessory proteins. Some examples of these are shown in yellow. **B.** Schematic diagram of the μ2-adaptin. Domains A (green) and B (blue) are comprised largely of β structures. Residues 102-125 (grey line) are implicated in binding leucine-based motifs ([DE]xxxL[LI]). Residues 283-394 (blue line) of domain B contain a region for binding basic-rich motifs. Residues 408-435 (green line) of domain A contain a critical region for binding a classical tyrosine motif (YxxØ).

β2, a medium-sized (50 kDa) adaptin, μ2, and a small (19 kDa) adaptin, σ2 (Fig. 5.1A; Hirst and Robinson, 1998; Collins *et al.*, 2002). The globular carboxyl-terminal regions of the α- and β2-adaptins form small appendages, which are each separated from the AP2 core by a proteolytically sensitive flexible linker (Fig. 5.1A; Collins *et al.*, 2002; Owen *et al.*, 2004; Kirchhausen *et al.*, 1989; Zaremba and Keen, 1985). The core region of AP2 forms multiple interactions with membrane proteins and lipids to recruit AP2 to the plasma membrane and for cargo recognition (Fig. 5.1A; Gaidarov and Keen, 1999; Chang *et al.*, 1993; Ohno *et al.*, 1995; Owen and Evans, 1998; Rapoport *et al.*, 1997; Rapoport *et al.*, 1998; Rodionov and Bakke, 1998). For example, the amino-terminus of the α-adaptin binds synaptotagmin (Haucke and De Camilli, 1999), PIP2 and PIP3 (Gaidarov *et al.*, 1996; Gaidarov and Keen, 1999), and the μ2-adaptin interacts with transmembrane cargo proteins (Ohno *et al.*, 1995; Owen and Evans, 1998; Rodionov and Bakke, 1998; Owen *et al.*, 2004) and PIP2 (Rohde *et al.*, 2002). The α and β2 appendages each bind several endocytic accessory factors, some of which are common to both of them (Fig. 5.1A). The α and β2 appendages both bind AP180, EPS15 and epsin 1 (Slepnev and De Camilli, 2000). The α appendage also interacts with amphiphysin (Slepnev *et al.*, 2000), and the β2 appendage with clathrin (Owen *et al*, 2000; Slepnev and De Camilli, 2000). A clathrin-binding sequence is also present in the β2 flexible linker (Owen *et al*, 2000).

Interestingly, GABA$_A$ receptors have been shown to form native complexes with AP2 and clathrin in brain (Kittler *et al.*, 2000a; Kumar *et al.*, 2003), and to co-localise with AP2 in primary cultures of hippocampal neurons (Kittler *et al.*, 2000a). The interaction between GABA$_A$ receptors and AP2 is mediated by μ2-adaptin, which binds directly to the major intracellular domain of the receptor β, γ2 and δ subunits (Kittler *et al.*, 2000a, 2005).

The μ2-adaptin consists of an amino-terminal domain, which comprises the first third of the protein, and a carboxyl-terminal domain, which comprises the remaining two thirds of the protein (Aguilar *et al.*, 1997; Owen and Evans, 1998; Collins *et al.*, 2002; Fig. 5.1B). The carboxyl-terminal domain can be further organised into two sub-domains, A and B, which are comprised largely of β-sheets (Fig. 5.1B; Owen and

Evans, 1998).

The μ2-adaptin recruits cargo proteins into clathrin-coated pits by recognising specialised endocytosis signals in their cytoplasmic domains. There are currently three major μ2-binding internalisation motifs: the tyrosine-based signals, the leucine-containing signals and the less well-characterised arginine/lysine-rich signals (or basic-rich motifs) (Fig. 5.1B). The tyrosine-based signals include YxxØ and NPxY (where x denotes a variable amino acid and Ø denotes a bulky hydrophobic residue (Leu, Ile, Met or Phe)), although the latter signal may also conform to FxNPxY (reviewed in Bonifacino and Traub, 2003; Jadot *et al.*, 1992; Boll *et al.*, 2002; Chen *et al.*, 1990). YxxØ signals mediate rapid internalisation from the plasma membrane, and are involved in the targeting of proteins to lysosomes (Williams and Fukuda, 1990; Harter and Mellman, 1992). These signals interact directly with the carboxyl-terminal domain of μ2-adaptin (Fig. 5.1B; Ohno *et al.*, 1995; Boll *et al.*, 1996; Ohno *et al.*, 1996; Owen and Evans, 1998), with residues D176 and W421 of μ2-adaptin being involved in the interaction (Nesterov *et al.*, 1999; Owen and Evans, 1998). NPxY motifs bind the μ2-adaptin at a site other than the YxxØ-binding region (Boll *et al.*, 2002; Warren *et al.*, 1998), and may interact with AP2 indirectly via other adaptor proteins (Mishra *et al.*, 2002; Nagai *et al.*, 2003).

The leucine-based signals take the form, [DE]xxxL[LI], where the residues four and five positions from the first leucine are typically, though not always, acidic, x denotes any amino acid, and the second leucine can be replaced by isoleucine or another hydrophobic residue (reviewed in Bonifacino and Traub, 2003). Leucine-based signals mediate rapid internalisation and endosomal/lysosomal targeting (Bonifacino and Traub, 2003). Evidence suggests that leucine-based motifs bind to the β2-adaptin of AP2 (Rapoport *et al.*, 1998), as well as to the μ2-adaptin at a site distinct from the YxxØ-binding region, between residues 102 and 125 of the amino-terminal domain (Fig. 5.1B; Bonifacino and Traub, 2003; Bremnes *et al.*, 1998; Rodionov and Bakke, 1998; Hofmann *et al.*, 1999; Marks *et al.*, 1996; Ohno *et al.*, 1995). However, direct interactions of this nature remain controversial (Bonifacino and Traub, 2003; Owen and Evans, 1998). The major intracellular domain of the GABA$_A$ receptor β2 subunit

contains a dileucine motif, which has been implicated in mediating GABA$_A$ receptor endocytosis in cortical neuronal slices and HEK-293 cells (Herring *et al.*, 2003, 2005). This motif is also present in the major intracellular domains of the receptor β1 and β3 subunits, but evidence suggests that it is not involved in the interaction of these subunits with the μ2-adaptin (Kittler *et al.*, 2005).

The non-canonical basic-rich motifs have only recently been identified in membrane proteins and receptors such as synaptotagmin 1 (Chapman *et al.*, 1998; Haucke *et al.*, 2000), the α1b subunit of the adrenergic receptor (Diviani *et al.*, 2003), the GluR2 subunit of the AMPA receptor (Lee *et al.*, 2002) and the β subunits of the GABA$_A$ receptor (Kittler *et al.*, 2005). These motifs have been shown to bind residues 283-394 of μ2-adaptin (Fig. 5.1B; Haucke *et al.*, 2000; Chapman *et al.*, 1998; Kittler *et al.*, 2005). In the GABA$_A$ receptor β subunits, the basic-rich motif is the sole site of μ2-adaptin binding, and encompasses the major sites of serine phosphorylation (Kittler *et al.*, 2005). Interestingly, μ2-adaptin binds to the basic-rich region in the β3 subunit in a phosphorylation-dependent manner (Kittler *et al.*, 2005). The μ2-adaptin displays high affinity for the dephosphorylated binding region, and peptide blockade of this interaction results in an enhancement in the amplitude of mIPSCs and whole-cell GABA$_A$ receptor currents, which has been attributed to a decreased rate of endocytosis and a corresponding increase in the number of cell-surface GABA$_A$ receptors (Kittler *et al.*, 2005).

Although GABA$_A$ receptors are known to interact with the AP2 complex, the physical relationship between AP2 and the GABA$_A$ receptor γ subunit family has not yet been fully described. In this chapter, I have taken a biochemical-based approach using affinity-purification assays, to investigate whether all members of the GABA$_A$ receptor γ subunit family bind directly to AP2, and to identify the AP2 adaptins that mediate such binding. I also aimed to identify the molecular determinants governing the interaction between AP2 and the γ2S subunit, and to investigate how this interaction is regulated. This approach has led to the discovery that GABA$_A$ receptor γ subunits each bind directly and selectively to the μ2-adaptin, and that the major intracellular domain of the γ2S subunit contains two distinct binding sites for μ2-

adaptin. I show that sub-domain B of μ2-adaptin binds to a putative, basic-rich region in the N-terminal half of the γ2S subunit, and that the YxxØ-interacting region of μ2-adaptin (in sub-domain A) binds to the C-terminal half of the γ2S subunit, which contains a classical tyrosine motif. I propose that these interactions facilitate the recruitment of synaptic GABA$_A$ receptors into clathrin-coated pits for internalisation, and that this is a key step in the regulation of postsynaptic GABA$_A$ receptor number and the efficacy of inhibitory synaptic transmission.

5.2 Results

An overview of the techniques used in this chapter is given in Figure 3.1.

5.2.1 The Major Intracellular Domain of Each GABA$_A$ Receptor γ Subunit Interacts with the Native, Neuronal AP2 Adaptor Complex

To test whether native AP2 interacts with all members of the GABA$_A$ receptor γ subunit family, I utilised affinity-purification assays and purified, bacterially expressed GST-fusion proteins of the major intracellular domain of GABA$_A$ receptor β3, γ1, γ2S, γ2L and γ3 subunits (GST-β3, GST-γ1, GST-γ2S, GST-γ2L and GST-γ3), as well as recombinant GST (Sections 2.2.4 and 2.5.11; Kittler *et al.*, 2005). The GST-fusion proteins were each exposed to a detergent-solubilised brain extract, and purified, together with any bound proteins, using glutathione-agarose beads. After resolving the protein complexes by SDS-PAGE, I probed for co-purified μ2-adaptin by Western blotting with a monoclonal anti-μ2 adaptin antibody (Sections 2.5.5 and 2.5.7; Table 2.6). I obtained a single 50-kDa immunoreactive band corresponding to μ2 adaptin in the affinity-purification input lane, and from affinity-purifications using GST-β3, GST-γ1, GST-γ2S, GST-γ2L and GST-γ3, but not GST alone (Fig. 5.2). These results demonstrated that the major intracellular domain of all members of the γ subunit family, and of the β3 subunit, bound either directly or indirectly to the native, neuronal AP2 complex. This suggested that neuronal AP2 binds non-selectively to GABA$_A$ receptor γ subunits, and that the major intracellular domain of each subunit is sufficient to mediate an interaction with AP2.

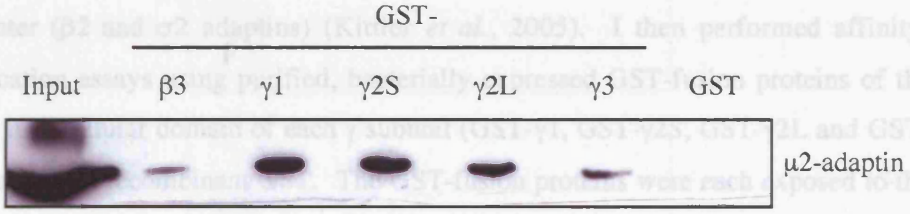


Figure 5.2. The major intracellular domains of $GABA_A$ receptor γ subunits bind to the μ2-adaptin of AP2 in brain. Affinity-purification assays were performed using GST-fusion proteins (20 μg) of the major intracellular domains of $GABA_A$ receptor γ1, γ2S, γ2L and γ3 subunits, as well as GST alone. GST-fusion proteins were exposed to a detergent-solubilised brain extract, and purified using glutathione-agarose beads. The protein complexes were then resolved by SDS-PAGE and probed for the μ2-adaptin by immunoblotting with a monoclonal anti-μ2 antibody, and an anti-mouse-HRP antibody for detection. 'Input' represents a proportion of the extract used in each binding assay.

5.2.2 The Major Intracellular Domain of Each GABA$_A$ Receptor γ Subunit Binds Directly and Selectively to the μ2-adaptin of the AP2 Adaptor Complex

I was next interested in determining whether the major intracellular domain of each GABA$_A$ receptor γ subunit interacts directly with the individual adaptins of the AP2 adaptor complex. To test for this, I utilised an *in vitro* protein expression system to transcribe and translate each of the two ~100 kDa AP2 adaptins, α and β2, the 50 kDa adaptin, μ2, and the 19 kDa adaptin, σ2 (Haucke *et al.*, 2000; Kittler *et al.*, 2005; Section 2.5.11.2; Table 2.2). This was done in the presence of [^{35}S]-labelled methionine, and under the control of the T7 promoter (α and μ2 adaptins) or the SP6 promoter (β2 and σ2 adaptins) (Kittler *et al.*, 2005). I then performed affinity-purification assays using purified, bacterially expressed GST-fusion proteins of the major intracellular domain of each γ subunit (GST-γ1, GST-γ2S, GST-γ2L and GST-γ3), as well as recombinant GST. The GST-fusion proteins were each exposed to the individual [^{35}S]-adaptins ([^{35}S]-α, [^{35}S]-β2, [^{35}S]-μ2 or [^{35}S]-σ2), and purified using glutathione-agarose beads. After the protein complexes were resolved by SDS-PAGE, each [^{35}S]-adaptin was visualised by phosphorimaging (Section 2.5.8). As shown in Figure 5.3, I detected a single band of 50 kDa corresponding to [^{35}S]-μ2 in the affinity-purification input lane, and from affinity-purification assays using GST-γ1, GST-γ2S, GST-γ2L and GST-γ3, but not GST alone. Interestingly, I also observed that the [^{35}S]-μ2 band was stronger from assays using GST-γ2L compared to GST-γ1, GST-γ2S and GST-γ3 (Fig. 5.3). Although I observed a single band at the expected molecular weight in the affinity-purification input lanes for [^{35}S]-α, [^{35}S]-β2, and [^{35}S]-σ2, I did not detect bands from the corresponding affinity-purification assays for any of the GST-γ fusion proteins tested or GST (Fig. 5.3). These data demonstrated that [^{35}S]-μ2, but not [^{35}S]-α, [^{35}S]-β2 or [^{35}S]-σ2, was co-purified with the major intracellular domain of each GABA$_A$ receptor γ subunit, *in vitro*, and that [^{35}S]-μ2 co-purified with GST-γ2L more than GST-γ1, GST-γ2S and GST-γ3. This suggested that the major intracellular domain of each GABA$_A$ receptor γ subunit binds directly and selectively to the [^{35}S]-μ2-adaptin of the AP2 complex, *in vitro*, and that the strongest interaction occurs between [^{35}S]-μ2 and the major intracellular domain of the γ2L subunit.

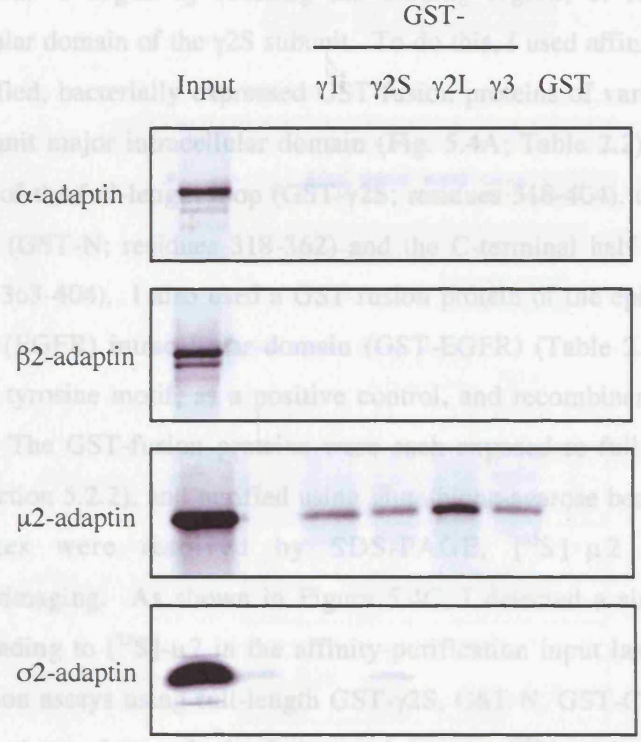


Figure 5.3. The major intracellular domains of $GABA_A$ receptor γ subunits bind selectively and directly to the μ2-adaptin of AP2. Each adaptin subunit (α, β2, μ2 and σ2) was *in vitro* transcribed and translated under the T7 promoter in the presence of [^{35}S]-labelled methionine. Affinity-purification assays were performed using GST-fusion proteins (20 μg) of the major intracellular domains of $GABA_A$ receptor γ1, γ2S, γ2L and γ3 subunits, as well as GST alone, and 5 μl of adaptin reaction mix. Proteins were resolved by SDS-PAGE, and [^{35}S]-adaptins were visualised by phosphorimaging. 'Input' represents 50% (v/v) of the [^{35}S]-adaptin reaction mix used in each assay.

5.2.3 The Major Intracellular Domain of the GABA$_A$ Receptor γ2S Subunit Contains Two Distinct Sites that each Bind Different Regions of the μ2-Adaptin

Having shown that the major intracellular domain of each GABA$_A$ receptor γ subunit interacts directly and selectively with the [^{35}S]-μ2-adaptin, I was interested in identifying the amino acid region, or regions, in each protein that is involved in the interaction. I began by locating the binding region, or regions, in the major intracellular domain of the γ2S subunit. To do this, I used affinity-purification assays and purified, bacterially expressed GST-fusion proteins of various fragments of the γ2S subunit major intracellular domain (Fig. 5.4A; Table 2.2). I used GST-fusion proteins of the full-length loop (GST-γ2S; residues 318-404), the N-terminal half of the loop (GST-N; residues 318-362) and the C-terminal half of the loop (GST-C; residues 363-404). I also used a GST-fusion protein of the epidermal growth factor receptor (EGFR) intracellular domain (GST-EGFR) (Table 2.2), which contains a classical tyrosine motif, as a positive control, and recombinant GST as a negative control. The GST-fusion proteins were each exposed to full-length [^{35}S]-μ2 (Fig. 5.4B; Section 5.2.2), and purified using glutathione-agarose beads. After the protein complexes were resolved by SDS-PAGE, [^{35}S]-μ2 was visualised by phosphorimaging. As shown in Figure 5.4C, I detected a single band of 50 kDa corresponding to [^{35}S]-μ2 in the affinity-purification input lane, and from affinity-purification assays using full-length GST-γ2S, GST-N, GST-C and GST-EGFR, but not GST alone. Interestingly, I observed that the [^{35}S]-μ2 band was stronger from assays using GST-N compared to GST-C (Fig. 5.4C). These data demonstrated that [^{35}S]-μ2 was co-purified with the major intracellular domain of the full-length GABA$_A$ receptor γ2S subunit, as well as each individual half of the major intracellular domain, *in vitro*, and that [^{35}S]-μ2 co-purified with GST-N more than GST-C. This suggested that the major intracellular domain of the GABA$_A$ receptor γ2S subunit contains at least two distinct *in vitro* binding regions for [^{35}S]-μ2, and that the site, or sites, in the N-half of the major intracellular domain mediates the strongest interaction.

To determine the region, or regions, of μ2 that binds to each of the γ2S subunit sites, I next transcribed and translated different fragments of the μ2 adaptin *in vitro*,

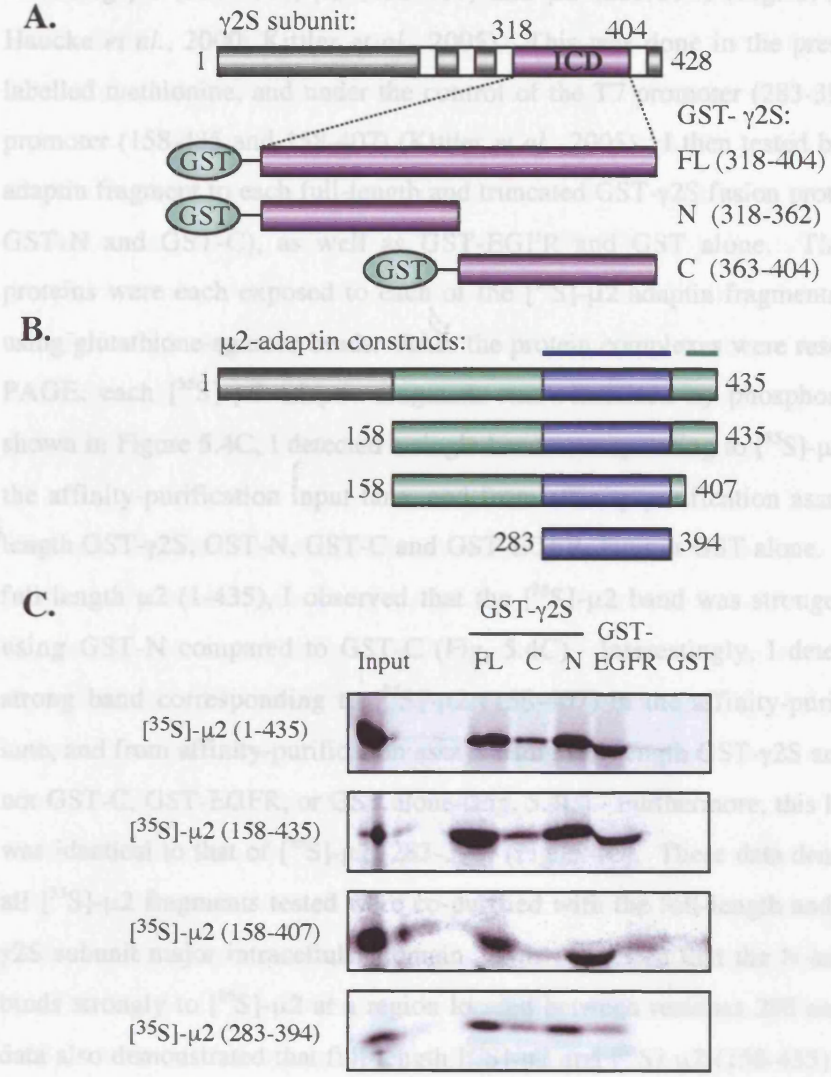


Figure 5.4. The major intracellular domain of the $GABA_A$ receptor γ2S subunit contains two distinct sites that each bind different domains of the μ2-adaptin. **A.** Schematic diagram of full-length (FL) GST-γ2S (318-404) and GST-γ2S deletion constructs (N: 318-362 and C: 363-404) used to map the binding site of μ2-adaptin. **B.** Schematic diagram of full-length μ2-adaptin (1-435) and μ2-adaptin deletion fragments (158-435, 158-407 and 283-394) used to map the binding site of the major intracellular domain (ICD) of the $GABA_A$ receptor γ2S subunit. Residues 407-435 (green line) contain a domain for binding a classical tyrosine motif. Residues 283-394 (blue line) contain a domain for binding basic-rich regions. **C.** Each adaptin construct (see 'B.') was *in vitro* transcribed and translated in the presence of [^{35}S]-labelled methionine. Affinity-purification assays were performed using the GST-γ2S fusion proteins (20 μg; see 'A.'), and GST alone, and 5 μl of adaptin reaction mix. Proteins were resolved by SDS-PAGE and [^{35}S]-adaptins were visualised by phosphorimaging. 'Input' represents 50% (v/v) of the [^{35}S]-adaptin reaction mix used in each assay.

including μ2 (158-435), μ2 (158-407) and μ2 (283-394) (Fig. 5.4B; Table 2.2; Haucke *et al.*, 2000; Kittler *et al.*, 2005). This was done in the presence of [^{35}S]-labelled methionine, and under the control of the T7 promoter (283-394) or the SP6 promoter (158-435 and 158-407) (Kittler *et al.*, 2005). I then tested binding of each adaptin fragment to each full-length and truncated GST-γ2S fusion protein (GST-γ2S, GST-N and GST-C), as well as GST-EGFR and GST alone. The GST-fusion proteins were each exposed to each of the [^{35}S]-μ2 adaptin fragments, and purified using glutathione-agarose beads. After the protein complexes were resolved by SDS-PAGE, each [^{35}S]-μ2 adaptin fragment was visualised by phosphorimaging. As shown in Figure 5.4C, I detected a single band corresponding to [^{35}S]-μ2 (158-435) in the affinity-purification input lane, and from affinity-purification assays using full-length GST-γ2S, GST-N, GST-C and GST-EGFR, but not GST alone. As shown for full-length μ2 (1-435), I observed that the [^{35}S]-μ2 band was stronger from assays using GST-N compared to GST-C (Fig. 5.4C). Interestingly, I detected a single, strong band corresponding to [^{35}S]-μ2 (158-407) in the affinity-purification input lane, and from affinity-purification assays using full-length GST-γ2S and GST-N, but not GST-C, GST-EGFR, or GST alone (Fig. 5.4C). Furthermore, this binding profile was identical to that of [^{35}S]-μ2 (283-394) (Fig. 5.4C). These data demonstrated that all [^{35}S]-μ2 fragments tested were co-purified with the full-length and N-half of the γ2S subunit major intracellular domain. This suggested that the N-half of the loop binds strongly to [^{35}S]-μ2 at a region located between residues 283 and 394. These data also demonstrated that full-length [^{35}S]-μ2 and [^{35}S]-μ2 (158-435), but not [^{35}S]-μ2 (158-407) or [^{35}S]-μ2 (283-394), co-purified with the C-half of the γ2S major intracellular domain, and the EGFR. This suggested that binding of the C-half of the loop, and the EGFR, to [^{35}S]-μ2 requires residues 408 to 435 of μ2-adaptin (a region known to bind tyrosine-based motifs (Owen and Evans, 1998; Nesterov *et al.*, 1999)), and that this interaction is weaker than that between the N-half of the loop and [^{35}S]-μ2. These data collectively suggest that the major intracellular domain of the γ2S subunit contains at least two distinct sites that each bind to different regions of [^{35}S]-μ2, *in vitro*.

Interestingly, residues 283-394 of μ2 have been shown to contain a domain for

binding basic-rich protein regions (Haucke *et al.*, 2000; Chapman *et al.*, 1998; Kittler *et al.*, 2005). Furthermore, analysis of the sequence of the N-half of the major intracellular domain of the γ2S subunit revealed that it contains such a putative region from residues 324 to 335 (Fig. 5.5A). Similarly, residues 408 to 435 of μ2 contain a domain for binding a tyrosine motif of the form YxxØ (Owen and Evans, 1998; Nesterov *et al.*, 1999), and analysis of the C-half of the major intracellular domain of the γ2S subunit reveals such a motif in the form YECL (Fig. 5.5A). These data therefore support binding of residues 283-394 of μ2 to the basic-rich region in the proximal region of the γ2S subunit major intracellular domain, and residues 408 to 435 of μ2 to the YECL motif in the more distal region of γ2S loop.

5.2.4 μ2-Adaptin Binds to a Putative Basic-Rich Region in the N-Terminus of the Major Intracellular Domain of the GABA$_A$ Receptor γ2S Subunit

To identify the region in the N-half (residues 318-362) of the major intracellular domain of the γ2S subunit that mediates binding to μ2-adaptin, I first generated a series of purified, bacterially expressed GST-γ2S fusion proteins containing N-terminal deletions at intervals of ~10 amino acids. These included GST-γ2S (327-404), GST-γ2S (338-404) and GST-γ2S (347-404) (Fig. 5.5A). The full-length (318-404) and truncated GST-γ2S fusion proteins were each exposed to [^{35}S]-μ2, and purified using glutathione-agarose beads. After the protein complexes were resolved by SDS-PAGE, [^{35}S]-μ2 was visualised by phosphorimaging. As shown in Figure 5.5B, I detected a single, strong band of 50 kDa corresponding to [^{35}S]-μ2 in the affinity-purification input lane, and from affinity-purification assays using full-length GST-γ2S. A weaker band was also seen from affinity-purification assays using GST-γ2S (327-404), GST-γ2S (338-404) and GST-γ2S (347-404) (Fig. 5.5B).

To determine whether each N-terminal truncation caused a significant loss of binding to [^{35}S]-μ2, I performed a quantitative analysis by measuring the intensity of each [^{35}S]-μ2 band using Bio-Rad Quantity One software (Sections 2.5.11.2 and 3.2.6.2). The band intensities obtained from the full-length and truncated GST-γ2S fusion proteins were each normalised to the input value, and expressed as a percentage. This was repeated for a number of individual experiments in order to calculate the mean

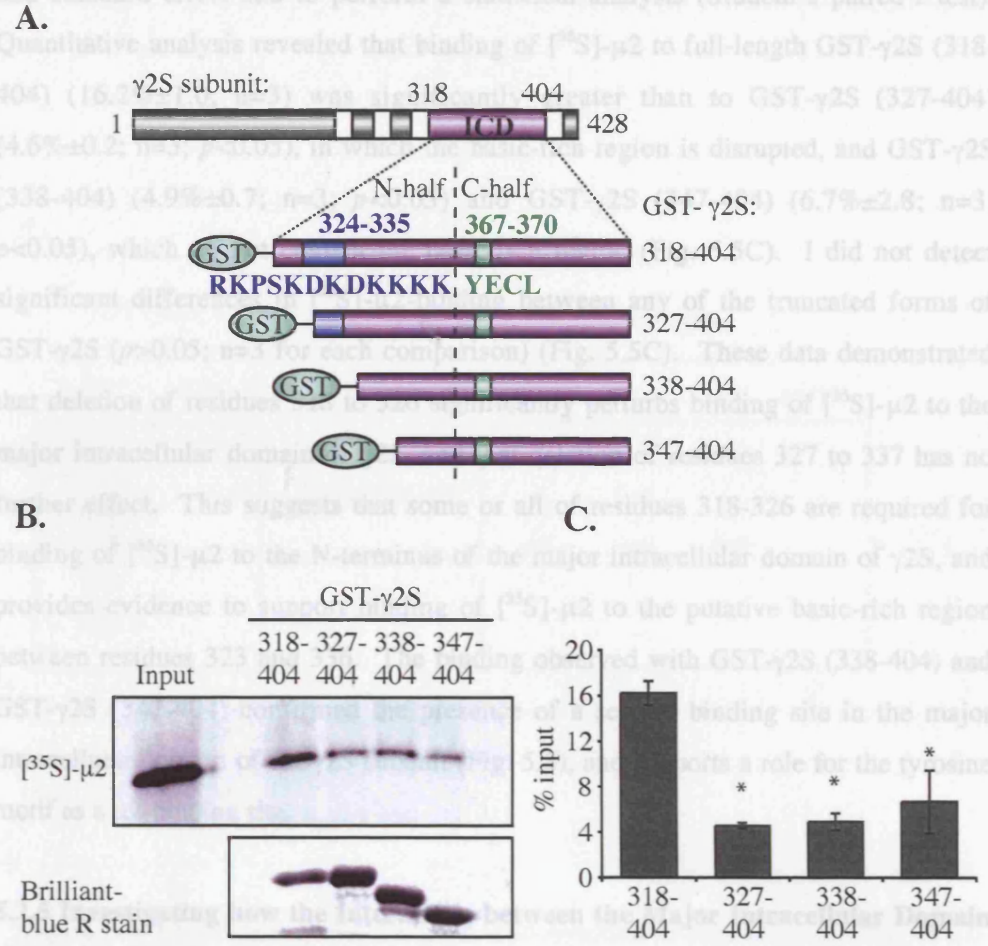


Figure 5.5. N-terminal deletions of the major intracellular domain of the γ2S subunit disrupt binding to μ2-adaptin. A. Schematic diagram of full-length and truncated GST-γ2S fusion proteins used to map the μ2-binding region in the N-half of the major intracellular domain of γ2S. The predicted μ2-binding regions in GST-γ2S are indicated, including an N-half basic-rich region in blue and a C-half tyrosine motif in green. **B.** Affinity-purification assays were performed using *in vitro* transcribed and translated [^{35}S]-μ2-adaptin and the GST-γ2S fusion proteins described in 'A.' (20 μg). After proteins were resolved by SDS-PAGE, [^{35}S]-μ2-adaptins were visualised by phosphorimaging. 'Input' represents 50% (v/v) of the [^{35}S]-adaptin reaction mix used in each assay. Brilliant-blue R stain showing equal-loading of the GST-fusion proteins is shown below. **C.** The magnitude of [^{35}S]-μ2-binding to each GST-fusion protein was expressed as a percentage of the input value. The mean and sem were calculated using the results of 3 independent experiments, and plotted in the chart shown. Differences between 318-404 and each truncation were analysed using the Student's paired *t* test. * denotes $p<0.05$.

and standard error, and to perform a statistical analysis (Student's paired *t* test). Quantitative analysis revealed that binding of [^{35}S]-μ2 to full-length GST-γ2S (318-404) (16.2%±1.0; n=3) was significantly greater than to GST-γ2S (327-404) (4.6%±0.2; n=3; $p<0.05$), in which the basic-rich region is disrupted, and GST-γ2S (338-404) (4.9%±0.7; n=3; $p<0.05$) and GST-γ2S (347-404) (6.7%±2.8; n=3; $p<0.05$), which do not contain the basic-rich region (Fig. 5.5C). I did not detect significant differences in [^{35}S]-μ2-binding between any of the truncated forms of GST-γ2S ($p>0.05$; n=3 for each comparison) (Fig. 5.5C). These data demonstrated that deletion of residues 318 to 326 significantly perturbs binding of [^{35}S]-μ2 to the major intracellular domain of γ2S, and that deletion of residues 327 to 337 has no further effect. This suggests that some or all of residues 318-326 are required for binding of [^{35}S]-μ2 to the N-terminus of the major intracellular domain of γ2S, and provides evidence to support binding of [^{35}S]-μ2 to the putative basic-rich region between residues 323 and 336. The binding observed with GST-γ2S (338-404) and GST-γ2S (347-404) confirmed the presence of a second binding site in the major intracellular domain of the γ2S subunit (Fig. 5.5), and supports a role for the tyrosine motif as a μ2-binding site.

5.2.5 Investigating how the Interaction between the Major Intracellular Domain of the γ2S Subunit and μ2-Adaptin is regulated

It has previously been shown that μ2-adaptin binds to a basic-rich region in the major intracellular domain of GABA$_A$ receptor β subunits, and that this interaction is blocked by phosphorylation of conserved serine residues that lie within the binding region (Kittler *et al.*, 2005). Interestingly, the putative basic-rich μ2-binding domain identified in the γ2S subunit contains a site of phosphorylation, at S327, for PKC (Moss *et al.*, 1992a; Kellenberger *et al.*, 1992; Krishek *et al.*, 1994). This residue is the only site in the γ2S subunit that is phosphorylated by PKC, and its phosphorylation state is involved in regulating the activity of recombinant and native GABA$_A$ receptors (Table 1.1; Moss *et al.*, 1992a; Kellenberger *et al.*, 1992; Krishek *et al.*, 1994). In this study, I wanted to determine whether phosphorylation of S327 regulates binding of μ2-adaptin to the γ2S subunit. To test this, I used purified PKC to phosphorylate GST-γ2S fusion proteins, *in vitro*. As shown in Figure 5.6A, I

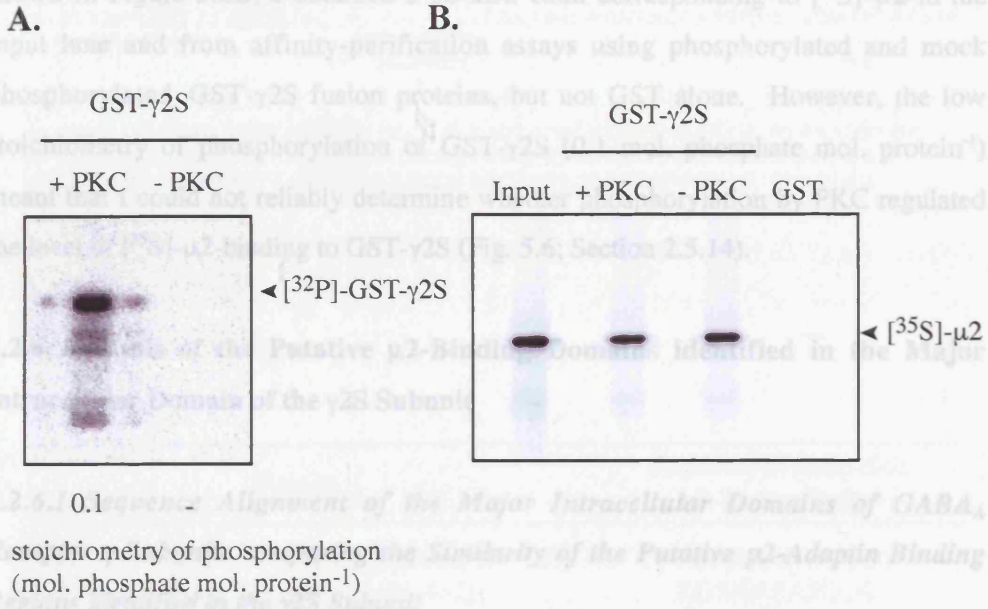


Figure 5.6. Investigating the role of phosphorylation in regulating binding of μ2-adaptin to the $GABA_A$ receptor γ2S subunit. A. Phosphorylation of GST-γ2S fusion proteins by PKC. The stoichiometry of phosphorylation (mol. phosphate mol. protein^{-1}) is given below each GST-fusion protein. **B.** Affinity-purification assays were performed using GST-γ2S fusion proteins that were phosphorylated on S327 by PKC, or mock phosphorylated (20 μg), or GST alone. The GST-fusion proteins were exposed to [^{35}S]-μ2-adaptin, and precipitated using glutathione-agarose beads. The protein complexes were then resolved by SDS-PAGE, and [^{35}S]-μ2-adaptin was visualised by phosphorimaging. 'Input' represents 10% (v/v) of the [^{35}S]-μ2 assay mix used in each binding assay.

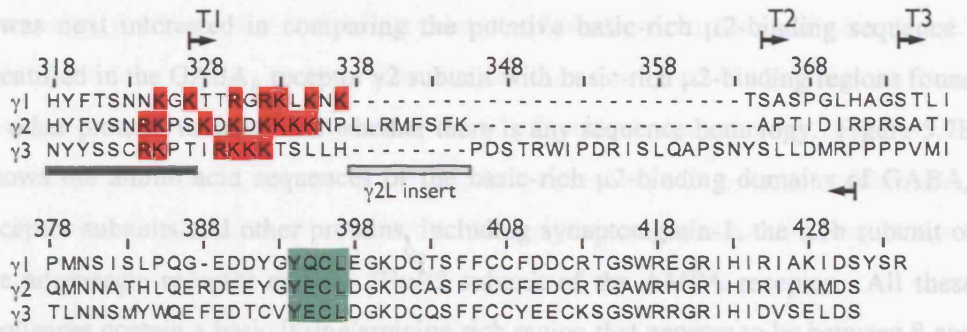
obtained a [^{32}P]-labelled, ~36 kDa protein band corresponding to phosphorylated GST-γ2S from the *in vitro* kinase assay performed in the presence of PKC, but not in its absence. I then performed affinity-purification assays using the phosphorylated/mock phosphorylated GST-γ2S fusion proteins and [^{35}S]-μ2. As shown in Figure 5.6B, I obtained a 50-kDa band corresponding to [^{35}S]-μ2 in the input lane and from affinity-purification assays using phosphorylated and mock phosphorylated GST-γ2S fusion proteins, but not GST alone. However, the low stoichiometry of phosphorylation of GST-γ2S (0.1 mol. phosphate mol. protein^{-1}) meant that I could not reliably determine whether phosphorylation by PKC regulated the level of [^{35}S]-μ2-binding to GST-γ2S (Fig. 5.6; Section 2.5.14).

5.2.6 Analysis of the Putative μ2-Binding Domains identified in the Major Intracellular Domain of the γ2S Subunit

5.2.6.1 Sequence Alignment of the Major Intracellular Domains of GABA$_A$ Receptor γ Subunits comparing the Similarity of the Putative μ2-Adaptin Binding Regions identified in the γ2S Subunit

To determine whether the putative μ2-adaptin binding sites identified in the major intracellular domain of the γ2S subunit (Figs. 5.4 and 5.5) are conserved in all the γ subunits, I constructed multiple sequence alignments of the γ subunit family. This was done using ClustalW (Higgins, 1994), and The Jalview Java Alignment Editor for manual editing (Clamp *et al.*, 2004). I observed conservation of a classical tyrosine motif in the C-terminal half of the major intracellular domain of all γ subunits (Fig. 5.7A). This motif takes the form YQCL in γ1, and YECL in γ2 and γ3 (Fig. 5.7A). I also observed arginine- and lysine-rich clusters between 9 and 13 amino acids in length at similar positions in the N-terminal half of the loops (Fig. 5.7A). These data demonstrated that the putative basic-rich and tyrosine-based μ2-adaptin-binding sites in the γ2S subunit are common to all the γ subunit isoforms. This suggested that μ2-adaptin associates with the major intracellular domain of each γ subunit by binding to a basic-rich region in the N-terminal half of the loop, and a tyrosine motif in the C-terminal half of the loop.

A.



B.

Protein	Sequence of Basic-Rich μ2-Binding Region
$GABA_A$ receptor γ2 subunit (putative; 324-335)	RKPSKDKDKKKK
$GABA_A$ receptor β1 subunit (401-412)	KGRIRRRASQLK
$GABA_A$ receptor β2 subunit (402-413)	KSRLRRRASQLK
$GABA_A$ receptor β3 subunit (401-412)	KTHLRRRSSQLK
synaptotagmin I C2B domain (321-332)	KRLKKKKTTIKK
Adrenergic receptor α1b subunit (371-378)	RRRRRRRR
AMPA receptor GluR2 subunit (844-853)	KRMKVAKNPQ

Figure 5.7. Sequence analysis of $GABA_A$ receptor γ subunits showing the position of the putative AP2 binding sites. A. The sequences shown represent the major intracellular domains of murine $GABA_A$ receptor γ1, γ2 and γ3 subunits. A black letter on a red box indicates a lysine or arginine residue that forms part of a basic-rich region. A black letter on a green box indicates a residues that forms part of a classical tyrosine motif (YxxØ). The light grey box indicates the sequence of the γ2L insert, and the dark grey box indicates a region containing residues that are crucial for binding of μ2 to the major intracellular domain of the γ2S subunit. The GST-γ2S truncations, T1 (327-404), T2 (338-404) and T3 (347-404) are indicated with arrows. Numerals represent the position of amino acids in the given alignment, and begin with respect to the γ2S subunit. **B.** Sequences of basic-rich μ2-binding regions identified in neuronal membrane proteins and receptors.

5.2.6.2 Comparison of the Basic-Rich μ2-Binding Region in the GABA$_A$ Receptor γ2 Subunit with similar μ2-Binding Regions in other Proteins

I was next interested in comparing the putative basic-rich μ2-binding sequence I identified in the GABA$_A$ receptor γ2 subunit with basic-rich μ2-binding regions found in other proteins to determine whether there is any sequence homology. Figure 5.7B shows the amino acid sequences of the basic-rich μ2-binding domains of GABA$_A$ receptor subunits and other proteins, including synaptotagmin-1, the α1b subunit of the adrenergic receptor and the GluR2 subunit of the AMPA receptor. All these sequences contain a basic lysine/arginine-rich region that appears to be between 8 and 12 residues in length (Fig. 5.7B). This further supports the finding of a basic-rich μ2-adaptin binding site in the N-terminus of the major intracellular domain of the GABA$_A$ receptor γ2 subunit.

5.3 Discussion

Regulating the number of GABA$_A$ receptors at mammalian central synapses is a key mechanism for controlling the efficacy of inhibitory synaptic transmission and neuronal excitability (Nusser *et al.*, 1997; Otis *et al.*, 1994; Nusser *et al.*, 1998b). GABA$_A$ receptors are constitutively internalised via a clathrin-mediated, dynamin-dependent pathway (Kittler *et al.*, 2000a, 2005). The association between the GABA$_A$ receptor and the AP2 adaptor complex is believed to play an important role in this process (Kittler *et al.*, 2000a, 2005). However, the physical interaction between these proteins has not yet been fully described. In this study, I investigated the interaction between GABA$_A$ receptor γ subunits and AP2, with the aim of further understanding the molecular mechanisms by which GABA$_A$ receptors are internalised from the cell surface. This study has provided evidence to suggest that the major intracellular domains of GABA$_A$ receptor γ subunits each bind selectively and directly to the μ2-adaptin of AP2 in brain. Mapping studies further revealed that the major intracellular domain of the γ2S subunit contains two distinct μ2-adaptin binding sites. I demonstrated that one of these sites is located in the N-terminus of the major intracellular domain, and comprises a putative basic-rich μ2-adaptin binding motif. This site was found to interact with sub-domain B of μ2-adaptin. A second site was located in the carboxyl-terminal half of the major intracellular domain, and likely

consists of a classical tyrosine-based μ2-adaptin binding motif. This site was found to interact with the carboxyl-terminus of μ2-adaptin, within sub-domain A. Interestingly, I found that both of the putative μ2-binding motifs identified in the γ2S subunit are common to all the γ subunit isoforms. I propose that μ2-adaptin forms multiple interactions with the receptor γ subunits, and that these facilitate the recruitment of GABA$_A$ receptors into clathrin-coated pits for internalisation. This mechanism may play an important role in regulating postsynaptic GABA$_A$ receptor number and hence the efficacy of inhibitory synaptic transmission.

My studies demonstrated that the major intracellular domain of each GABA$_A$ receptor γ subunit is able to form a complex with the native neuronal AP2 complex. They also revealed that the GABA$_A$ receptor γ subunits each bind selectively to the μ2-adaptin of the AP2 complex. This is consistent with previous studies demonstrating that the receptor β subunits also bind selectively to μ2-adaptin (Kittler *et al.*, 2005). Given the importance of μ2-adaptin in cargo recognition (Ohno *et al.*, 1995; Owen and Evans, 1998; Rodionov and Bakke, 1998), this suggests that binding of receptor γ subunits (and β subunits) to μ2-adaptin plays an important role in the selective translocation of GABA$_A$ receptors into clathrin-coated pits. It is perhaps not surprising that the σ2 subunit did not bind directly to the γ subunits, as this adaptin appears to have a structural role within the AP2 complex (Collins *et al.*, 2002). Interestingly, the membrane protein synaptotagmin is the only protein so far identified that interacts with both μ2- and α-adaptin (Haucke *et al.*, 2000). Although synaptotagmin binds independently to each adaptin, it has been suggested that binding is co-operative (that binding to one adaptin enhances binding to the other and, hence, to the AP2 complex) (Haucke *et al.*, 2000). It is therefore possible that GABA$_A$ receptors do not associate with multiple adaptins because co-operative binding is achieved through the multivalent interactions μ2-adaptin forms with the different subunits (β, γ and δ) that co-assemble to form a receptor complex. I shall return to this concept later in the discussion.

I also observed enhanced binding of μ2-adaptin to the major intracellular domain of the γ2L subunit compared to the other γ subunit isoforms. This may be due to the

presence of two consecutive leucine residues in the γ2L insert sequence, LLRMFSFK, which may form part of a consensus leucine-based μ2-binding motif, [DE]xxxL[LI] (see Section 5.1). However, evidence suggests that leucine-based motifs bind to the μ2- and β-adaptins (Rapoport *et al.*, 1998; Rodionov and Bakke, 1998), and I did not detect an interaction of γ2L with β2-adaptin. Furthermore, the dileucine motif in the receptor β1 and β3 subunits is not involved in the binding of either of these subunits to the μ2-adaptin (Kittler *et al.*, 2005). The increased affinity of μ2-adaptin for the γ2L subunit may alternatively be explained by a conformational change that is a consequence of the eight amino acid insertion, and which results in enhanced access of μ2-adaptin to the tyrosine-based and basic-rich binding sites.

The mapping experiments in this study revealed that the major intracellular domain of the γ2S subunit contains two distinct μ2-binding sites that each recognise different regions of the μ2-adaptin. The evidence suggested that the μ2-binding site in the amino-terminal half of the loop comprises a putative basic-rich motif consisting of up to eight arginine/lysine amino acids (residues 324-335), and that R324 and/or K325 are critical residues of this motif. This was based on the finding that binding of μ2-adaptin to the γ2S subunit loop was significantly reduced by N-terminal loop deletions that disrupted or removed the basic-rich motif. Further support was obtained from the finding that this N-terminal loop region interacted with residues 283 to 394 of μ2-adaptin. This region of μ2-adaptin has previously been shown to interact with a basic-rich motif in the β subunits of GABA$_A$ receptors (Kittler *et al.*, 2005), the synaptic vesicle protein synaptotagmin (Haucke *et al.*, 2000; Chapman *et al.*, 1998), and the α1b subunit of the adrenergic receptor (Diviani *et al.*, 2003). The amino acid similarity of the basic-rich motifs in these proteins and the receptor γ subunits, and the ability of the γ subunit major intracellular domains to each bind native neuronal AP2 complexes, suggests that the putative γ2S basic-rich motif has a conserved role in binding the AP2 complex in neurons.

My studies also supported the idea that μ2-adaptin binds to a second distinct site in the carboxyl-terminal half of the γ2S subunit major intracellular domain, which comprises a classical tyrosine-based motif of the form, YECL (residues 367 to 370).

This is based on the finding that binding of the C-terminal half of the γ2S subunit, like the EGFR, to μ2-adaptin was significantly disrupted by deletion of the carboxyl-terminal region of μ2-adaptin. Although co-crystallisation studies have shown that both D176 and W421 of sub-domain A of μ2-adaptin are involved in binding tyrosine-based (YxxØ) internalisation signals (Owen and Evans, 1998), mutational analysis and affinity-purification experiments with the EGFR showed that while D176A partially inhibits μ2-adaptin binding, W421A completely abrogates binding (Nesterov *et al.*, 1999). Consistent with this, I found that the full-length and carboxyl-terminal half of the γ2S subunit major intracellular domain, like the EGFR, only interacted with μ2-adaptin deletion constructs that contained W421, suggesting that the YECL motif of the γ2S subunit binds to a region of μ2-adaptin containing W421. It is possible, that like the EGFR, this interaction is not essential for clathrin-dependent internalisation of GABA$_A$ receptors (Nesterov *et al.*, 1999). Indeed, this study has identified two distinct μ2-adaptin binding sites in the γ2S subunit.

It has previously been shown that association of AP2 with synthetic peptides containing a YQRL endocytic motif induces a transition of AP2 from an inactive to an active state, which results in an increased binding capacity for the cargo protein synaptotagmin (Haucke and De Camilli, 1999; Owen and Evans, 1998). It is therefore possible that binding of μ2-adaptin to the tyrosine motif in the γ2S subunit increases the interaction of μ2-adaptin with GABA$_A$ receptors. As μ2-adaptin binds to the N-half binding site with greater affinity than to the C-half binding site, it is possible that the weak interaction with the tyrosine motif induces a conformational change that enables a stronger interaction to take place with the basic-rich motif. As previously mentioned in this discussion, co-operativity of binding of AP2 to GABA$_A$ receptors may also involve binding sites found on other receptor subunits. Furthermore, it is possible that binding of the AP2 complex to GABA$_A$ receptor subunits modifies the binding properties of clathrin/endocytic accessory proteins. The presence of multiple μ2-adaptin binding sites in GABA$_A$ receptor subunits and the presence of binding co-operativity may enable neurons to regulate the rate of internalisation of distinct GABA$_A$ receptor populations.

It would be interesting to investigate the role of individual μ2-adaptin binding sites in the internalisation of neuronal $GABA_A$ receptors in functional studies. Electrophysiological and biochemical experiments using synthetic peptides mimicking the putative μ2-adaptin binding sites identified in γ2S could help identify any co-operativity of binding, and to determine the importance of each site in the process of internalisation, and in the control of postsynaptic $GABA_A$ receptor number. As the putative basic-rich and tyrosine-based motifs identified in this study are common to all the γ subunit isoforms, such peptides could also be used to investigate the role of these sites in mediating binding of μ2-adaptin to the γ1 and γ3 subunits, and the endocytosis of $GABA_A$ receptors that contain these subunits.

In this study, I attempted to investigate whether the interaction between the γ2S subunit and μ2-adaptin was regulated by phosphorylation. My approach was similar to that used by Kittler and colleagues (Kittler *et al.*, 2005) to examine the phospho-dependence of μ2-adaptin binding to the receptor β subunits. In this study, I utilised purified PKC to phosphorylate S327, which lies within the putative basic-rich region of the γ2S subunit. Unfortunately, the low stoichiometry of phosphorylation prevented me from accurately assessing the level of μ2-adaptin binding to the phosphorylated and mock-phosphorylated subunit forms. This is because the ratio of phosphorylated to mock-phosphorylated GST-fusion protein was very low, which meant that even if phosphorylation completely inhibited binding, it would only block a small proportion of the total μ2-adaptin binding. Furthermore, as there are two distinct μ2-adaptin binding sites in the major intracellular domain of the γ2S subunit, then binding may not be completely abrogated by blockade of the interaction with the basic-rich region. However, given that phosphorylation of the basic-rich region in the receptor β3 subunit inhibited binding of μ2-adaptin (Kittler *et al.*, 2005), it is likely that phosphorylation of the putative basic-rich region in the γ2S subunit would have a similar effect. Fortunately, this hypothesis can be tested by performing [^{35}S]-μ2-adaptin binding assays with synthetic peptides of the putative basic-rich binding motif, that are chemically phosphorylated or mock-phosphorylated at S327.

Phosphorylation of Y367 of the putative tyrosine-based motif in the γ2S subunit may

also regulate binding of μ2-adaptin. Phosphorylation of the tyrosine in YxxØ binding motifs has previously been shown to interfere with binding of μ2-adaptin (Ohno *et al.*, 1996; Owen and Evans, 1998). Y367 of the γ2S subunit has also been shown to be a site of phosphorylation for the tyrosine kinase Src *in vitro* and in heterologous cells (Moss *et al.*, 1995; Valenzuela *et al.*, 1995), and is constitutively phosphorylated in brain (Brandon *et al.*, 2001). Phosphorylation of Y367 is known to result in enhanced receptor function due to an increase in the probability of channel opening (Moss *et al.*, 1995). It would therefore be interesting to determine whether phosphorylation of this residue also perturbs binding of μ2-adaptin to regulate the number of receptors on the cell surface and, hence, GABA$_A$ receptor-mediated currents.

In addition to phosphorylation, the binding of AP2 to GABA$_A$ receptor γ subunits may also be regulated by the interactions of these subunits with other cellular proteins. For example, binding of Src and PP2B to the γ2 subunit is believed to involve amino acids 317 to 332 (Wang *et al.*, 2003a; Sections 1.7.2.1.7 and 1.7.2.2.3), and may therefore involve residues of the putative basic-rich μ2-adaptin binding site. Furthermore, the binding site for the palmitoyl acyl transferase, GODZ, in the γ2 subunit is believed to lie within amino acids 368 and 381 (Keller *et al.*, 2004; Section 1.6), and may therefore encompass some residues of the putative tyrosine-based μ2-adaptin binding motif. It is also possible that the palmitoylation of cysteine residues within this GODZ-binding region (Keller *et al.*, 2004; Rathenberg *et al.*, 2004; Section 1.6) also regulates the interaction between the GABA$_A$ receptor γ2 subunit and AP2.

5.4 Conclusions

Overall, I conclude that the GABA$_A$ receptor γ subunits each bind directly and selectively to the μ2-adaptin of AP2, and to the native AP2 complex in brain. The γ2S subunit contains at least two distinct binding sites for μ2-adaptin, which are located in the N- and C-terminal halves of the major intracellular domain. The N-half binding motif comprises a putative basic-rich region, and interacts with sub-domain B of μ2-adaptin. The C-terminal binding motif likely comprises a tyrosine-based

interaction motif, and interacts with the C-terminus of μ2-adaptin, within sub-domain A. The putative basic-rich and tyrosine-based μ2-adaptin binding motifs are common to all the γ subunit isoforms. The multivalent interactions between μ2-adaptin and the receptor γ subunits may underlie the ability of the AP2 complex to associate with $GABA_A$ receptors at the neuronal surface. This may facilitate the selective recruitment of $GABA_A$ receptors into clathrin-coated pits for internalisation, and be part of a molecular mechanism controlling the number of cell-surface $GABA_A$ receptors. The interaction between AP2 and $GABA_A$ receptor γ subunits may therefore have significant consequences for the efficacy of inhibitory synaptic transmission.

CHAPTER 6

General Discussion

6.1 Biological and Clinical Implications of the Physical and Functional Interaction between CaMKII and the $GABA_A$ Receptor

The phosphorylation state of the $GABA_A$ receptor is believed to be an important determinant of channel activity and the efficacy of inhibitory neurotransmission (Moss and Smart, 2001; Poisbeau *et al.*, 1999; Section 1.7). A number of signalling pathways leading to changes in the phosphorylation state of $GABA_A$ receptors have now been described (Brandon *et al.*, 2002; Jovanovic *et al.*, 2004; Terunuma *et al.*, 2004; Wang *et al.*, 2003b), and the precise means by which kinases are targeted to $GABA_A$ receptor scaffolds are beginning to emerge (Brandon *et al.*, 1999, 2001, 2002, 2003; Jovanovic *et al.*, 2004). Although several lines of evidence suggest that CaMKII is involved in regulating $GABA_A$ receptor function in neurons (Aguayo *et al.*, 1998; Wang *et al.*, 1995; Kano *et al.*, 1996; Section 3.1), the molecular mechanism of such regulation remains to be elucidated. In Chapters 3 and 4 of this thesis, I provided evidence for a model whereby activity-dependent recruitment of CaMKII to $GABA_A$ receptors is involved in regulating subunit phosphorylation (Fig. 6.1).

The data obtained in Chapter 3 revealed that CaMKIIα interacts physically with $GABA_A$ receptors in brain. Further investigations demonstrated that native CaMKIIα binds to the major intracellular domain of various receptor subunits without any apparent selectivity. The interaction between CaMKIIα and the intracellular domains of the receptor β subunits was found to be dependent upon phosphorylation of the kinase at T286, but appeared to be independent of $GABA_A$ receptor subunit phosphorylation. Indeed, a major binding site for CaMKII was located within the first 19 amino acids of the major intracellular domain of the receptor β subunit, at least 60 residues upstream of the CaMKII *in vitro* phosphorylation sites. These results suggested that neuronal stimuli that lead to an increase in intracellular calcium concentration and autophosphorylation of CaMKII at T286 could trigger binding of the kinase to $GABA_A$ receptors (Fig. 6.1). If this is the case, then this phosphorylation-dependent interaction may underlie the targeting of CaMKII to $GABA_A$ receptors in neurons. Furthermore, it may facilitate activity-dependent phospho-modulation of $GABA_A$ receptor activity and hence the efficacy of inhibitory

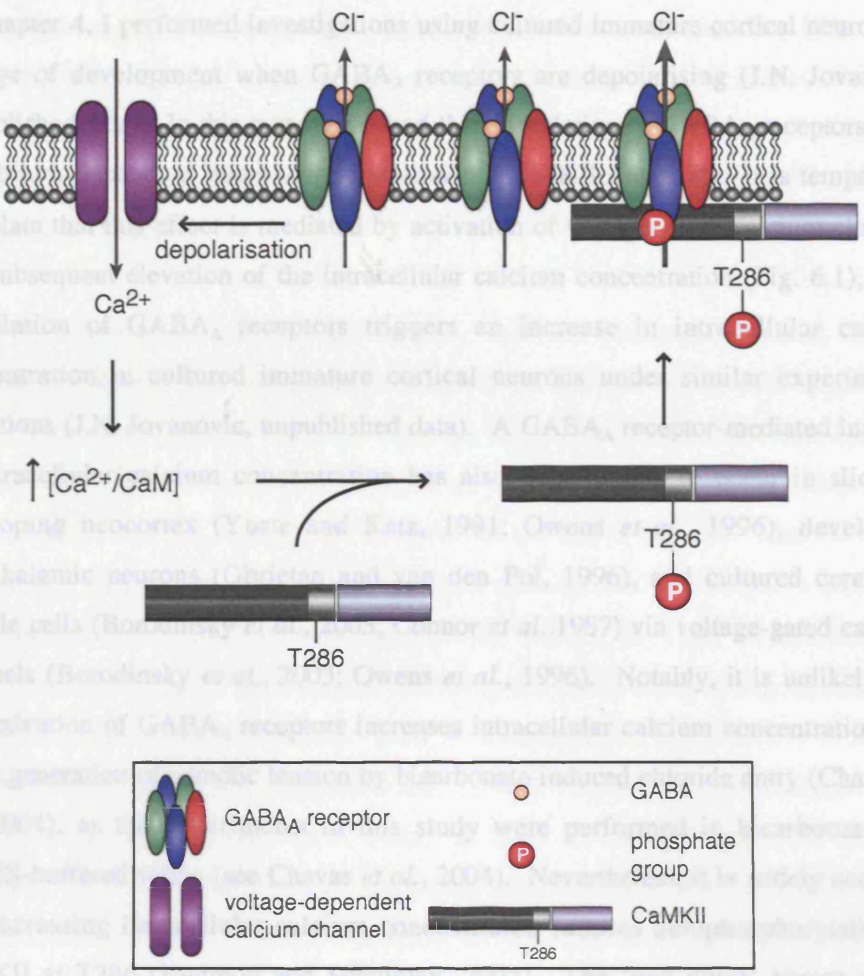


Figure 6.1. Proposed model of CaMKII-dependent phosphorylation and functional modulation of $GABA_A$ receptors in immature cortical neurons. Stimulation of $GABA_A$ receptors triggers neuronal depolarisation and a rise in intracellular calcium concentration (J.N. Jovanovic, unpublished data), which may be mediated by voltage-dependent calcium channels. This is proposed to result in T286-autophosphorylation of CaMKII, and subsequent binding of the active kinase to $GABA_A$ receptors. CaMKII then phosphorylates specific sites within the major intracellular domains of particular receptor subunits to modulate $GABA_A$ receptor activity. This mechanism may provide positive feedback signalling to $GABA_A$ receptors during neuronal development as CaMKII is believed to enhance GABA-mediated currents in cortical neurons (Aguayo *et al.*, 1998).

synaptic transmission.

In Chapter 4, I performed investigations using cultured immature cortical neurons, at a stage of development when $GABA_A$ receptors are depolarising (J.N. Jovanovic, unpublished data). In this system, I found that stimulation of $GABA_A$ receptors leads to calcium-dependent autophosphorylation of CaMKIIα at T286. It is tempting to speculate that this effect is mediated by activation of voltage-gated calcium channels and subsequent elevation of the intracellular calcium concentration (Fig. 6.1), since stimulation of $GABA_A$ receptors triggers an increase in intracellular calcium concentration in cultured immature cortical neurons under similar experimental conditions (J.N. Jovanovic, unpublished data). A $GABA_A$ receptor-mediated increase in intracellular calcium concentration has also been shown to occur in slices of developing neocortex (Yuste and Katz, 1991; Owens *et al.*, 1996), developing hypothalamic neurons (Obrietan and van den Pol, 1996), and cultured cerebellar granule cells (Borodinsky *et al.*, 2003; Connor *et al.* 1987) via voltage-gated calcium channels (Borodinsky *et al.*, 2003; Owens *et al.*, 1996). Notably, it is unlikely that the activation of $GABA_A$ receptors increases intracellular calcium concentration due to the generation of osmotic tension by bicarbonate-induced chloride entry (Chavas *et al.*, 2004), as the experiments in this study were performed in bicarbonate-free HEPES-buffered saline (see Chavas *et al.*, 2004). Nevertheless, it is widely accepted that increasing intracellular calcium concentration induces autophosphorylation of CaMKII at T286 (Hudmon and Schulman, 2002). The 'pull-down'-kinase assays performed in this chapter further demonstrated that depolarisation of cultured immature cortical neurons increases the functional interaction of CaMKII with the major intracellular domain of the $GABA_A$ receptor β3 subunit. These findings collectively reinforce the view that depolarisation of cultured immature cortical neurons could induce docking of T286-phosphorylated CaMKII to $GABA_A$ receptors (Fig. 6.1).

The data obtained in Chapter 4 revealed that purified CaMKII selectively phosphorylates the major intracellular domains of the $GABA_A$ receptor β and γ subunits, but not the α or δ subunits, *in vitro*, and identified the major intracellular

domains of the γ1 and γ3 subunits as novel substrates of CaMKII. The results also confirmed that S383 and S409 are the *in vitro* phosphorylation sites of CaMKII in the receptor β3 subunit (McDonald and Moss, 1997), and demonstrated that PP1, PP2A and PP2C, but not PP2B, dephosphorylate the β3 subunit, *in vitro*, at these CaMKII sites. These findings provided preliminary evidence that S383 and S409 of the β3 subunit may be important sites of $GABA_A$ receptor phosphorylation-dependent modification in neurons.

A previous study has shown that CaMKII does not phosphorylate the $GABA_A$ receptor β3 subunit in cultured immature cortical neurons under basal conditions (Brandon *et al.*, 2000). In this chapter, I revealed that muscimol-induced depolarisation of cultured immature cortical neurons triggers CaMKII-dependent phosphorylation of the receptor β3 subunit, but not the γ2 subunit. Chapter 4 also documented attempts to raise phosphorylation state-specific antibodies to phospho-S383 and phospho-S409 of the receptor β3 subunit in order to examine the phosphorylation status of each residue following muscimol-induced depolarisation of cultured immature cortical neurons. This work exemplified the difficulty in raising such biological tools, and suggested that CaMKII-dependent phosphorylation of $GABA_A$ receptors does not occur solely at S409 in cultured immature cortical neurons. Moreover, the results suggested that CaMKII may be involved in phosphorylation of both S408 and S409, and did not exclude the possibility that S383 is a major site of CaMKII phosphorylation in neurons.

It is tempting to speculate from the data obtained in Chapters 3 and 4 that muscimol-induced depolarisation of immature cortical neurons triggers CaMKII-dependent phosphorylation of $GABA_A$ receptors via differential targeting of the activated kinase to receptor complexes (Fig. 6.1). The translocation of CaMKII to $GABA_A$ receptors could enable a highly regulated response to local changes in calcium concentration driven by activation of $GABA_A$ receptors. This mechanism could mediate positive or negative feedback signalling to $GABA_A$ receptors during the early stages of neuron development, when GABA is excitatory. Translocation of CaMKII to $GABA_A$ receptors could trigger a change in the activity and/or number of postsynaptic

$GABA_A$ receptors, and therefore alter the strength and/or stability of GABAergic synapses, and inhibit or promote subsequent postsynaptic calcium entry.

Phosphorylation of $GABA_A$ receptors is believed to have important functional consequences for channel activity and neuronal excitability (Moss and Smart, 2001; Poisbeau *et al.*, 1999; Section 1.7). Evidence suggests that the effect of phosphorylation on $GABA_A$ receptor functionality is dependent upon several factors, including cell type, subunit composition, and the identity of the kinase (Moss and Smart, 2001; Section 1.7). A number of studies now suggest that activation of CaMKII leads to an enhancement in $GABA_A$ receptor activity. In cortical neurons, an increase in intracellular calcium concentration leads to a CaMKII-dependent increase in the amplitude of GABA-mediated currents (Aguayo *et al.*, 1998). Potentiation of GABA-mediated currents is also observed in cortical neurons following intracellular application of pre-activated CaMKII (catalytic and regulatory domains) (C.M. Houston, unpublished data). These results are consistent with findings from other cell-types. Addition of pre-activated CaMKII to cerebellar granule cells increases the amplitude of GABA-mediated currents (C.M. Houston, unpublished data), and increases the amplitude and decay time of IPSCs (C.M. Houston, unpublished data). Addition of CaMKII also potentiates GABA-induced currents in spinal dorsal horn neurons, and evoked IPSPs in hippocampal CA1 neurons (Wang *et al.*, 1995). Moreover, intracellular application of purified, active CaMKII to cerebellar Purkinje neurons enhances the amplitude of both GABA-mediated currents and spontaneous IPSCs (Kano *et al.*, 1996).

It is possible that CaMKII-dependent potentiation of $GABA_A$ receptor activity in neurons is a result of direct phosphorylation of receptor subunits. If so, then the data obtained in Chapters 3 and 4 suggest that the receptor β3 subunit could play an important role in such functional modulation. This is supported by recent investigations showing that intracellular application of pre-activated CaMKII increases the amplitude of GABA-mediated currents in NG108-15 cells expressing α1/β3 subunits and α1/β3/γ2 subunits, whereas the effect is abolished in cells expressing α1/β3(S383A) subunits, and significantly reduced in cells expressing

α1/β3(S383A)/γ2 subunits (C.M. Houston, unpublished data). Furthermore, addition of pre-activated CaMKII has no effect in cells expressing α1/β2/γ2, and only a small effect in cells expressing α1/β1/γ2 (which is mediated via the γ2 subunit) (C.M. Houston, unpublished data). These results therefore suggest that CaMKII-dependent functional modulation of $GABA_A$ receptors is mediated, at least in part, by S383 of the β3 subunit. This is consistent with the finding that KCl-induced depolarisation of NG108-15 cells induces phosphorylation of the $GABA_A$ receptor β3 subunit at S383 (C.M. Houston, unpublished data). It is therefore tempting to speculate that in cultured immature cortical neurons, CaMKII-dependent phosphorylation of $GABA_A$ receptor β3 subunits occurs at S383 and enhances $GABA_A$ receptor activity, therefore contributing to a positive feedback-signalling pathway to $GABA_A$ receptors during neuronal development (Fig. 6.1). This could have a profound effect on the strength of GABAergic synapses, and hence the input-output relationship of neurons and the excitability of neural networks, as well as GABA-mediated calcium-dependent signalling events.

The physical and functional interaction of CaMKII with $GABA_A$ receptors may mediate long-term changes in the strength of inhibitory synapses (reviewed by Gaiarsa *et al.*, 2002) during neuronal maturation and/or in adult neurons, which have important consequences for the excitability of neurons and neural circuits, network physiology and animal behaviour. Long-term potentiation (LTP) and long-term depression (LTD) of GABAergic synapses has been detected in various brain regions, including the cortex, hippocampus, cerebellum, lateral superior olive, deep cerebellar nucleus and brainstem (Gaiarsa *et al.*, 2002). Indeed, in the developing hippocampus, stimulation of $GABA_A$ receptors has been shown to trigger membrane depolarisation, an increase in intracellular calcium concentration, via activation of voltage-gated calcium channels, and LTP of $GABA_A$ receptors (Caillard *et al.*, 1999; Gubellini *et al.*, 2001; Gaiarsa *et al.*, 2002). Furthermore, in cerebellar Purkinje neurons, CaMKII has been implicated in the induction of a long-lasting rebound potentiation of GABAergic synapses in which activation of glutamatergic inputs leads to a calcium-dependent increase in $GABA_A$ receptor currents (Kano *et al.*, 1992, 1996; Kawaguchi and Hirano, 2002).

The results from Chapters 3 and 4 may have some clinical relevance as both CaMKII and the $GABA_A$ receptor β3 subunit have been implicated in the neurodevelopmental disorder, Angelman syndrome (AS). AS is characterised by severe mental retardation, epilepsy, motor impairment, sleep disturbances and craniofacial abnormalities (Williams *et al.*, 1995; Buoni *et al.*, 1999; Laan *et al.*, 1999). AS results from various genetic abnormalities of chromosome region 15q11-13, such as maternal deletion, uniparental paternal disomy, imprinting defects and mutations within the UBE3A gene, which encodes an E6-P ubiquitin ligase (Jiang *et al.*, 1999). Although in a small number of cases, no genetic abnormality has been identified (Jiang *et al.*, 1999; Clayton-Smith and Laan, 2003). Investigations of a mouse model for AS, which was generated by a *Ube3a* maternal null mutation, have revealed deficits in hippocampal LTP and context-dependent learning that are thought to involve altered autophosphorylation and activity of CaMKII (Jiang *et al.*, 1998; Weeber *et al.*, 2003). However, in addition to *Ube3a*, chromosome region 15q11-13 also contains a cluster of genes encoding the $GABA_A$ receptor β3, α5 and γ3 subunits, namely GABRB3, GABRA5 and GABRG3 (Glatt *et al.*, 1997). These genes are widely expressed in the developing mammalian brain (Laurie *et al.*, 1992b), and mutant mice deficient in gabrb3 exhibit a number of phenotypic characteristics resembling AS (Homanics *et al.*, 1997; DeLorey *et al.*, 1998). Indeed, aberrant function of GABRB3 has been implicated in the severe epilepsy seen in AS patients with deletion of chromosome region 15q11-13 (Minassian *et al.*, 1998). It is therefore possible that dysfunctional CaMKII-dependent phosphorylation of $GABA_A$ receptors is partially responsible for the AS phenotype.

6.2 Biological and Clinical Implications of the Interaction between the AP2 Adaptor Complex and the $GABA_A$ Receptor

In addition to their functional properties, the total number of postsynaptic $GABA_A$ receptors also determines the efficacy of inhibitory synaptic transmission (Otis *et al.*, 1994; Nusser *et al.*, 1998b; Wan *et al.*, 1997b; Wang *et al.*, 2003b; Brunig *et al.*, 2001; Jovanovic *et al.*, 2004). Neuronal $GABA_A$ receptors are cycled constitutively between the plasma membrane and intracellular compartments (Connolly *et al.*, 1999a,b; Kittler *et al.*, 2000a,b, 2004a, 2005). Internalisation of $GABA_A$ receptors

occurs via a clathrin-mediated, dynamin-dependent pathway (Kittler *et al.*, 2000a, 2005; Tehrani and Barnes, 1993, 1997; Tehrani *et al.*, 1997), and the interaction between the $GABA_A$ receptor and the AP2 adaptor complex is believed to play an important role in this process (Kittler *et al.*, 2000a; Kumar *et al.*, 2003). In Chapter 5 of this thesis, I identified the molecular determinants of the interaction between the AP2 adaptor complex and the $GABA_A$ receptor γ subunits.

The data from Chapter 5 revealed that the major intracellular domain of each $GABA_A$ receptor γ subunit binds the native AP2 complex in brain. Further analysis using *in vitro* binding assays demonstrated that each $GABA_A$ receptor γ subunit interacts directly and selectively with the μ2-adaptin of AP2. The γ2S subunit was shown to contain at least two distinct binding sites for μ2-adaptin in the N- and C-terminal halves of the major intracellular domain. The N-half of γ2S interacted with sub-domain B of μ2-adaptin via a putative basic-rich binding motif. The C-half of γ2S interacted with the C-terminus of μ2-adaptin, within sub-domain A, and this was proposed to occur via a tyrosine-based binding motif. The construction of multiple sequence alignments revealed that the putative basic-rich and tyrosine-based μ2-adaptin binding motifs are common to all the γ subunit isoforms.

It is possible that the multivalent interactions between each $GABA_A$ receptor γ subunit and μ2-adaptin underlie the ability of AP2 to associate with $GABA_A$ receptors at the neuronal surface. This may facilitate the selective recruitment of $GABA_A$ receptors into clathrin-coated pits for internalisation, and therefore be part of a molecular mechanism involved in maintaining the number of cell-surface $GABA_A$ receptors and hence the strength of GABAergic synapses, neuronal excitability and the dynamics of neural networks. The interactions between AP2 and the $GABA_A$ receptor γ subunits may also be the target of intracellular signalling pathways that modify constitutive rates of $GABA_A$ receptor endocytosis and therefore the stability of $GABA_A$ receptors at the neuronal surface. Furthermore, as $GABA_A$ receptors undergo constitutive and agonist-dependent endocytosis (Kittler *et al.*, 2000a, 2005; Connolly *et al.*, 1999a,b; Calkin and Barnes, 1994; Calkin *et al.*, 1994; Tehrani and Barnes, 1991), it is important to consider the role of agonists and allosteric

modulators in the internalisation process. For example, the $GABA_A$ receptor γ subunit (predominantly γ2) appears to be involved in the physical and functional interaction between $GABA_A$ receptors and benzodiazepines (Pritchett *et al.*, 1989; Smith and Olsen, 1995). It is therefore possible that the multivalent interactions between the AP2 complex and the $GABA_A$ receptor γ subunits are involved in the development of tolerance and dependence to benzodiazepines, a phenomenon that is known to limit the therapeutic use of these clinically active compounds.

6.3 Future Directions

Future research will inevitably extend our current knowledge of mechanisms that maintain or change the phosphorylation state and cell-surface stability of $GABA_A$ receptors. Throughout this thesis, I have commented on a number of steps that may be taken to further delineate the putative mechanisms described in this body of work. In the medium-term, it may be of interest to investigate additional effects of $GABA_A$ receptor activation during neuronal maturation. This could involve identifying other downstream effectors of CaMKII, as there is some evidence that this kinase is involved in the trophic effects of GABA during neuronal development (Borodinsky *et al.*, 2003). Future research may also involve investigating whether upstream regulators of CaMKII, such as calmodulin, bind to and regulate $GABA_A$ receptors. It may also be of interest to further examine the potential role of CaMKII-dependent binding and phosphorylation in long-term plasticity at GABAergic synapses, such as in rebound potentiation, and in controlling the cell-surface stability of $GABA_A$ receptors. New insights into $GABA_A$ receptor trafficking may be obtained from work examining the functional role of the multiple AP2 binding sites in $GABA_A$ receptor subunits and complexes. Additional research may also determine whether $GABA_A$ receptor endocytosis occurs via a non-clathrin route, and identify further proteins and subunit modifications that are involved in mediating or regulating the internalisation of $GABA_A$ receptors.

In the longer term, the generation of knockin mice may also provide key insight into the phospho-modulation and cell-surface stability of $GABA_A$ receptors. The introduction of point mutations into the mouse genome that mimic or prevent

phosphorylation at particular subunit sites combined with biochemical and electrophysiological experiments could not only confirm the sites of kinase activity *in vivo*, but the effects of phosphorylation on channel activity and cell-surface stability, and the signalling pathways that regulate this post-translational modification. This approach may also indicate whether developing and mature neurons use similar mechanisms to regulate the phosphorylation state of $GABA_A$ receptors. Knockin mice could also be used to characterise and confirm sequences involved in the binding of protein kinases and phosphatases to $GABA_A$ receptors, and to determine whether binding is a pre-requisite of subunit phosphorylation or other functional effects. Mutant mice could also be used to identify and confirm sequences involved in the trafficking of $GABA_A$ receptors to and from the neuronal surface.

It will also be interesting to investigate the interplay of protein kinases and phosphatases at $GABA_A$ receptor scaffolds. This will involve examining the binding dynamics of the various enzymes and their anchoring proteins, and other signalling components that may have similar or overlapping binding regions. In addition, it will involve extending our knowledge of where phosphorylation modifies binding of signalling molecules, and determining whether synergistic phosphorylation of different subunit sites occurs. The numerous phosphorylation sites and binding partners of a given $GABA_A$ receptor complex, together with the potential interplay between them, provides great potential for there being a large number of distinct functional states of the receptor. Combined with the heterogeneity of $GABA_A$ receptors, this could generate a wide range of responses. It will therefore be of interest to determine the cellular processes that limit such potential complexity, such as the spatio-temporal control of the expression, alternative splicing, sub-cellular targeting and stability of $GABA_A$ receptor-associated proteins. The complexity generated from the potential interplay of signalling molecules at $GABA_A$ receptors could contribute to the remarkable specificity of signalling responses to various extracellular stimuli. Furthermore, this complexity will continue to shape the intellectual and experimental challenge of elucidating the cellular, physiological and behavioural significance of neuronal signalling events that lead to changes in the

phosphorylation state and cell-surface stability of $GABA_A$ receptors in health and disease.

APPENDIX

Publications and
Conference Abstracts

Research Article:

Kittler, J. T., Chen, G., Honing, S., Bogdanov, Y., **McAinsh, K.**, Arancibia-Carcamo, I. L., Jovanovic, J. N., Pangalos, M. N., Haucke, V., Yan, Z., and Moss, S. J. (2005). Phospho-dependent binding of the clathrin AP2 adaptor complex to GABAA receptors regulates the efficacy of inhibitory synaptic transmission. Proc Natl Acad Sci U S A 102, 14871-14876.

Review Article:

Kittler, J. T., **McAinsh, K.**, and Moss, S. J. (2002). Mechanisms of GABAA receptor assembly and trafficking: implications for the modulation of inhibitory neurotransmission. Mol Neurobiol 26, 251-268.

Conference Abstracts:

Kittler, J. T., Chen, G., Jovanovic, J. N., **McAinsh, K.**, Bogdanov, Y., Haucke, V., Yan, Z., and Moss, S. J. (2004). Regulation of inhibitory synaptic transmission by phospho-dependent regulation of the association of GABA-A receptors and the AP2 adaptin complex. Soc for Neurosci Abs San Diego CA USA.

Jovanovic, J. N., **McAinsh, K.**, Longbottom, R., Sihra, T. S., and Moss, S. J. (2004). Neurotrophin-dependent regulation of GABA-A receptor internalization in developing cerebrocortical neurons. Soc for Neurosci Abs San Diego CA USA.

McAinsh, K. (2004). CaM kinase II-dependent phosphorylation of $GABA_A$ receptors in cortical neurons. Final Year Students' Meeting. The Wellcome Trust London.

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